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STOMACH WORM (HAEMONCHUS CONTORTUS) INFECTION IN LAMBS AND ITS RELATION TO GASTRIC HEMORRHAGE AND GENERAL PATHOLOGY [1]

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INTRODUCTION

The life history of the stomach worm, *Haemonchus contortus*, was first worked out experimentally by Ransom (*7*) [2] in 1906. Nine years later Veglia (*8*) made a thorough study of this nematode, including observations on the pathological changes produced by it in the abomasum of experimentally infected lambs. He found that the worms caused considerable hemorrhage into the stomach during the fourth and fifth stages of their life cycle.

In 1931 Fourie (*4*) made the first detailed study of the pathology and hematology of *H. contortus* infection in sheep. He concluded that the anemia observed in sheep experimentally infected with this nematode was purely hemorrhagic in character, since he was able to reproduce the same blood picture in healthy lambs by periodic bleeding from the jugular vein. Three years later, Martin and Clunies Ross (*6*) made a "minimal" estimate of the quantity of blood withdrawn daily by *H. contortus* females. These authors calculated the quantity of phosphorus in the eggs laid by the worms, and, on the assumption that this phosphorus came wholly from the blood of the host, they estimated that 2,000 female worms would withdraw about 30 cc. of blood daily. They concluded that the total loss of blood as a result of such an infestation could safely be doubled, since the 30 cc. did not include the blood consumed by the male worms, that which undoubtedly passed through the alimentary tract of the worms unchanged, and that which was lost by hemorrhage into the stomach. They agreed with Fourie that the effects of heavy infestations with *H. contortus* were due to the continua loss of blood, without any additional action of toxic secretions or products of the parasite's metabolism.

Boughton and Hardy (*1*) were able to observe the behavior of this parasite through fistulas made in the fourth stomach of infected sheep. They found that the parasites attached themselves to the stomach wall by a peculiar striking motion of the head and neck; that they remained

[1] Received for publication November 6, 1941.

[2] Italic numbers in parentheses refer to Literature Cited, p. 17.

attached for about 12 minutes and sucked blood, which could be followed through the intestine of the nematode almost to the anus; and that minute hemorrhages, which continued for a maximum of 7 minutes, occurred as soon as the worms detached themselves from the stomach wall.

It is evident from the literature cited that *H. contortus* infection in sheep may cause severe anemia in these animals, but except for the report of Boughton and Hardy there is no direct evidence that this anemia is the result of hemorrhage.

It is the purpose of the present paper to present additional evidence to the effect that (1) infection of lambs with the common sheep stomach worm, *H. contortus*, may result in severe and even fatal anemia; (2) this anemia is chiefly due to hemorrhage into the fourth stomach; (3) the quantity of blood lost by the hemorrhage may be estimated by determining the quantity of blood passed in the feces; (4) the anemia produced by stomach worm infection is similar to that caused by the periodic withdrawal of measured quantities of blood by bleeding from the jugular vein; and (5) the anemia is the result, at least in part, of blood loss due to continuous, seeping, capillary hemorrhage resulting from injury caused by the worms to the mucous membrane of the wall of the stomach.

MATERIALS AND METHODS

Nineteen lambs 2 to 8 months of age were used in these experiments, which were conducted in 1933 and from 1936 to 1938, inclusive. All the lambs were born and raised at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md., and were Hampshire-Southdown crosses. They were kept in cages having concrete floors, which were cleaned daily. The lambs were fed an adequate ration consisting of alfalfa hay and a grain mixture. Salt and water were kept before them at all times. At the beginning of the experiments the lambs were free of all nematode parasites except *Strongyloides papillosus*, which apparently did not affect the results of the experiments, since this parasite was present to the same extent in both control and infected lambs.

For the purpose of infecting the experimental lambs, female specimens of *Haemonchus contortus* were obtained from freshly slaughtered sheep at an abattoir near Washington, D. C. The eggs from these worms were cultured in helminthologically sterile sheep feces and animal charcoal, and the infective larvae were administered per os to a worm-free lamb by means of a funnel and rubber tube. When this lamb began to pass eggs of *H. contortus*, the feces were cultured with animal charcoal, and the larvae obtained were administered to the experimental lambs in the doses shown in table 1.

Whenever possible the length of time between the first dose of infective larvae and the first appearance of blood and stomach worm eggs in the feces was ascertained. At frequent intervals blood samples were taken from the jugular vein of all lambs, and records were made of the total number of red and white cells per cubic millimeter of blood, the number of grams of hemoglobin per 100 cc. of blood, the percentage of packed red cells, by volume, in 1 cc. of whole blood (hereafter referred to as volume percentage of packed red cells), the percentage of the

TABLE 1.—*Data on degree of infection of lambs with Haemonchus contortus and first appearance of blood and worm eggs in feces*

Lamb No.	Approximate age at beginning of infection	Date of administration of larvae	Larvae per dose	Doses	Total larvae	Period between first dose of larvae and appearance of—		Date of post mortem examination	Period between first dose of larvae and post mortem examination	Adult worms or larvae recovered
						Blood in feces	Worm eggs in feces			
	Mos.	*1933*	*Number*	*Number*	*Number*	*Days*	*Days*	*1933*	*Days*	*Percent*
1	2.5	May 18 June 27 July 20	[1] 500 30,000 45,000	1 1 1	[1] 75,500	.....	27	Aug. 1	75	26.36
		1936						*1936*		
2	2.0	May 7	2,000	1	2,000	.....	18	Dec. 9	216	.....
3	4.0	July 6–Aug. 30 Sept. 1–25	1,000 5,000	56 25	181,000	.....	14	Oct. 30	116	.38
4	4.0	July 8–Aug. 30 Sept. 1	500 1,000	84 1	43,000	.....	18	....do	114	.86
5	3.0	July 13	35,000	1	35,000	.....	18	Aug. 27	45	4.47
6	6.0	Sept. 8–Oct. 7	5,000	31	155,000	.....	22	Oct. 30	52	2.13
								1937		
7	8.0	Dec. 23	45,000	1	45,000	6	.....	Oct. 25	306	.002
		1937								
8	8.0	Jan. 6	13,000	1	13,000	10	.....	Jan. 27	21	4.53
9	6.0	Aug. 26	8,800	1	8,800	8	18	Dec. 10	106	.....
10	6.0	Sept. 17–18	[1] 2,500	2	[1] 5,000	6	.....	Sept. 25	8	[1] 10.00
		1938						*1938*		
11	3.0	July 30	100,000	1	100,000	10	.....	Aug. 22	23	4.77
12	3.0	July 30–Aug. 23	5,000	25	125,000	10	.....	Aug. 24	25	[1] 4.00
13	4.0	Aug. 27	70,000	1	70,000	.....	.....	Aug. 31	4	3.14
14	4.0	....do	70,000	1	70,000	6	.....	Sept. 3	7	[1] 2.85
15	4.0	....do	70,000	1	70,000	7	.....	Sept. 7	11	[1] 2.85
16	4.0	....do	70,000	1	70,000	.....	.....	Sept. 19	23	[1] 2.85

[1] Approximate.

different types of white cells, and the number of reticulocytes per 1,000 red cells. The number of worm eggs per gram of feces passed by the infected lambs was also ascertained at frequent intervals. Observations on the icteric index were made, but since there were no deviations from normal these data are omitted.

The presence of blood in the feces was determined by the benzidine test. A Levy-Hausser counting chamber was used for obtaining the total red and white cell counts. A clinical model Haden-Hausser hemoglobinometer was used for the hemoglobin determinations, and a Wintrobe hematocrit was used for ascertaining the volume percentage of packed red cells. The blood smears from which the differential white cell counts were made were stained with Wright's stain. For the reticulocyte counts, the blood was stained in vitro with brilliant cresyl blue. Approximately 500 white cells and 1,000 red cells were counted for the two determinations.

In order to ascertain the relationship between the different stages of development of the worms within the host and the pathological changes produced in the abomasum, four lambs (Nos. 13, 14, 15, and 16) were given equal numbers of infective larvae on the same day and were then killed at different intervals after infection.

The total quantity of blood passed with the feces of two lambs (Nos. 11 and 12) during the period that they harbored fatal infections

of *H. contortus* was ascertained by a modification of the method published by Van Eck (*2*) for determining the quantity of blood in human feces. One gram of sheep feces was boiled with three separate 20-cc. portions of glacial acetic acid. The acid from the three extractions was filtered off by suction through a piece of No. 1 Whatman filter paper placed over a Büchner funnel, and was collected in a large test tube. The filtrate was then thoroughly mixed, measured, and a small known fraction of the total, the size of which depended on the quantity of blood in the sample, was treated with a 1-percent solution of commercial benzidine in glacial acetic acid and 3 percent hydrogen peroxide. After dilution with 95 percent alcohol and 5 percent sodium hydroxide in distilled water, the resulting solution was placed in a colorimeter and the color compared with that of a similarly treated standard containing a known quantity of blood. As a control on this phase of the work, a known quantity of blood was removed daily, except Sundays, from the jugular vein of lamb 17, and blood counts made to determine the degree of anemia produced. Lambs 18 and 19 also were uninfected and used as controls in determining the changes in red cells and hemoglobin in the blood.

DATA OBTAINED

WORMS RECOVERED AND FIRST INDICATIONS OF BLOOD AND EGGS IN FECES

The data on the degree of infection of the experimental lambs with *Haemonchus contortus* and the first appearance of blood and worm eggs in the feces are given in table 1.

Lambs 1, 11, and 12 died of *H. contortus* infection. Lamb 5 was apparently in the last stages of the disease, as is indicated in figure 4, when it was killed for post mortem examination. The remainder of the lambs recovered from the effects of the experimental infections administered and were autopsied on the dates indicated.

In those lambs in which blood or worms eggs appeared in the feces, the former was observed, on the average, about 8 days after infection and the latter about 20 days after. In lambs 11 and 12 death occurred before worm eggs appeared in the feces, thus making a positive diagnosis of *H. contortus* infection impossible prior to post mortem examination. There was a delay of a few days in the appearance of worm eggs in the feces of lambs 1 and 6. In lamb 1 this delay was no doubt due to the very large infestation present, resulting in an overcrowded condition and a retardation of development of the worms. In lamb 6, the delay was probably due to the fact that the lamb was able to build up some resistance to the infection as it had received comparatively large daily doses of larvae for a considerable period.

The percentages of larvae that were able to establish themselves in the host animals varied. However, since the interval between infection and necropsy also varied, it was impossible to determine whether there was a significant difference between the number of worms establishing themselves in the lambs that received only a few doses and in those that received daily doses for a fairly long period of time.

RED CELLS, HEMOGLOBIN, AND BLOOD RECOVERED IN FECES

Figures 1 to 8 show, for both experimental and control lambs, the changes in the number of red cells per cubic millimeter of blood, the

number of grams of hemoglobin per 100 cc., the volume percentage of packed red cells, and the number of worm eggs per gram of feces. Figures 5, 6, and 7 also contain data on the quantity of blood lost by lambs 11, 12, and 17, respectively.

Lambs Recovering From Infection

Data on the red-cell counts and hemoglobin were obtained on all the lambs that recovered from the infection, but the results from only three representative lambs (Nos. 4, 3, and 9) are presented in this paper.

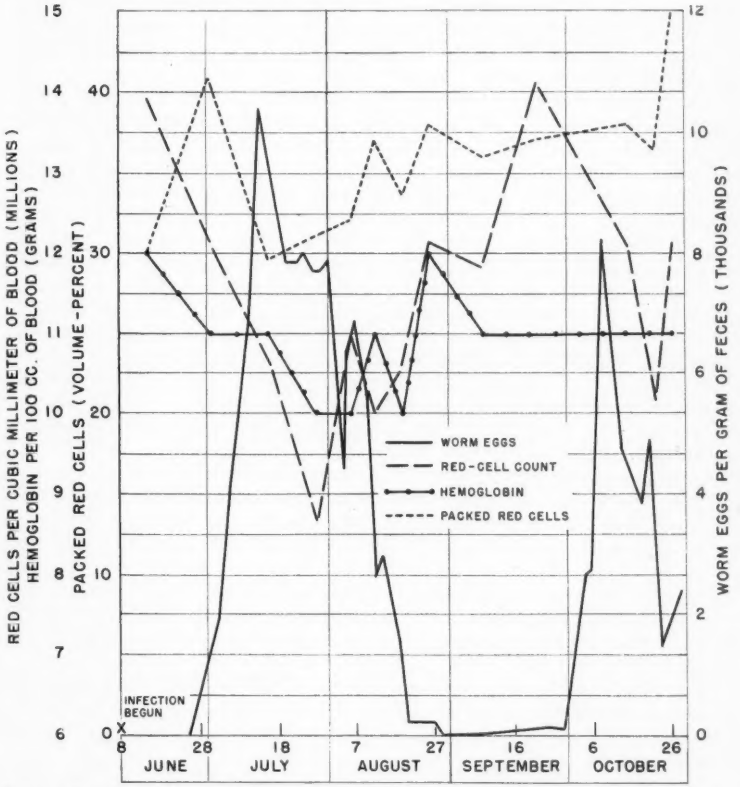
RED CELLS PER CUBIC MILLIMETER OF BLOOD (MILLIONS)
HEMOGLOBIN PER 100 CC. OF BLOOD (GRAMS)
PACKED RED CELLS (VOLUME-PERCENT)
WORM EGGS PER GRAM OF FECES (THOUSANDS)
WORM EGGS
RED-CELL COUNT
HEMOGLOBIN
PACKED RED CELLS
INFECTION BEGUN
8 28 18 7 27 16 6 26
JUNE JULY AUGUST SEPTEMBER OCTOBER

FIGURE 1.—Red cells and hemoglobin in the blood of lamb 4 correlated with the number of worm eggs per gram of feces passed during experimental infection with *Haemonchus contortus*.

The daily doses of larvae administered to lamb 4 (fig. 1) were apparently too small to produce marked symptoms of stomach worm disease, as is indicated by the relatively small decrease in the number of red cells per cubic millimeter of blood. Although the number decreased to 8.6 millions, it was not sufficient to cause marked changes in the hemoglobin or in the volume percentage of packed red cells.

The most significant observation recorded in figure 1 is the correlation between the low point of the red-cell curve and the high point of the worm-egg curve. Following the first peak in the egg count, the number of eggs decreased rapidly to almost zero, in spite of the daily administration of larvae. Simultaneously, there occurred a rapid increase in the number of red cells and in the hemoglobin. Approximately 5 weeks later there was a second increase in the number of worm eggs with a second slight decrease in the number of red cells, the two phenomena occurring again with approximately the same time relation between them. These data show a definite negative correlation between the number of worm eggs and the number of red cells in the blood. They also indicate that lamb 4 developed a partial resistance against superinfection, only to have it broken down temporarily a short time later by continued reinfection.

The greater number of larvae administered to lamb 3 caused a correspondingly greater decrease in the number of red cells, quantity of hemoglobin, and volume percentage of packed red cells (fig. 2) than in the case of lamb 4. In lamb 3 the number of red cells decreased to 6.5 millions per cubic millimeter of blood shortly after the number of eggs per gram of feces reached its maximum of 12,700. In this lamb also a sudden decrease occurred in the number of eggs per gram of feces in spite of continual superinfection, and there was also a simultaneous rapid increase in the number of red cells, quantity of hemoglobin, and volume percentage of packed red cells. As with lamb 4, a secondary increase in the number of eggs in the feces occurred simultaneously with a slight decrease in the number of red cells.

In lamb 9 (fig. 3), the changes brought about in the blood by the worm infection paralleled closely those in lambs 4 and 3. However, in lamb 9, the peak of the worm-egg curve reached 24,800 eggs per gram of feces, whereas the lowest point of the red-cell curve was 4.2 millions per cubic millimeter of blood. The low points of the curves for hemoglobin and for the volume percentage of packed red cells were reached about a month later than that for the number of red cells. Coincident with the decrease in egg count was the characteristic rapid recovery of the blood to normal within about 29 days. The peak of the egg-count curve of this lamb was much higher and broader than that of the two preceding lambs, owing probably to the fact that lamb 9 had no chance through superinfection to develop resistance against the infection.

In all three lambs that recovered from the infection, there was found to be a negative correlation between the egg count and (1) the number of red cells per cubic millimeter of blood and (2) the quantity of hemoglobin per 100 cc.

Lambs Dying From Infection

In the study of the blood picture of the lambs that died of the infection, lamb 5 also was included since, as already stated, it apparently was in the last stages of the disease when it was killed for post mortem examination. Complete data on lamb 1 were not available; consequently, this lamb was omitted from the study.

The data for lamb 5 are given in figure 4. The blood picture of this animal differs from that of the foregoing ones in that there was no

tendency for the number of red cells and quantity of hemoglobin to return to normal with a decrease in the number of eggs per gram of feces. It also differs from the others in that the decrease in the number of red cells occurred before the peak of the egg count had been reached.

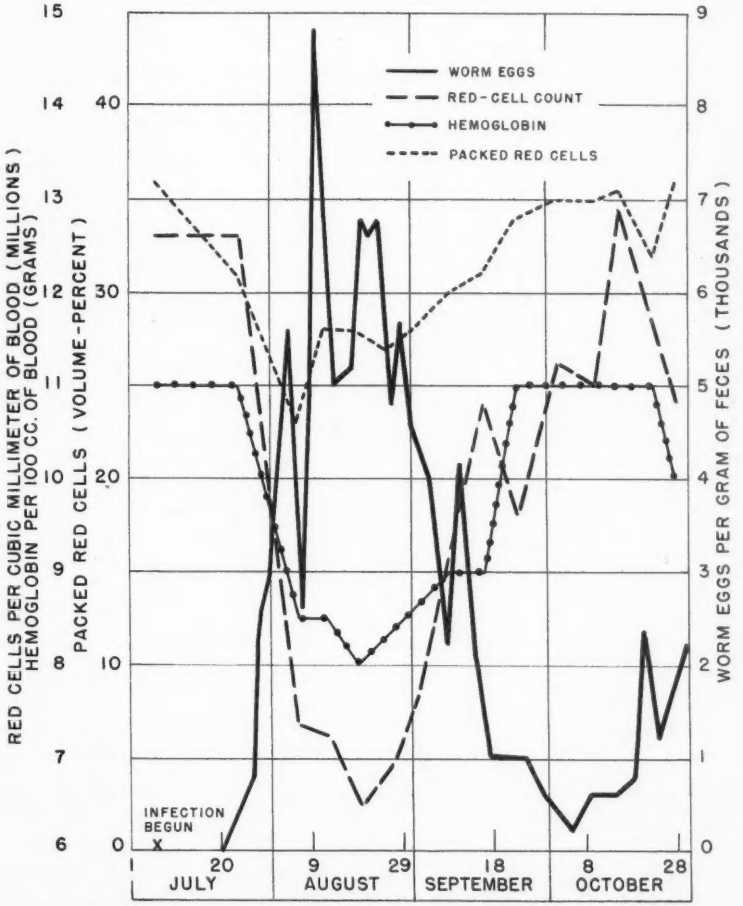

FIGURE 2.—Red cells and hemoglobin in the blood of lamb 3 correlated with the number of worm eggs per gram of feces passed during experimental infection with *Haemonchus contortus*.

In fact, most of this decrease occurred before the number of eggs per gram of feces rose to a figure that would be diagnostic of stomach worm disease. This lamb was anesthetized with ether and the stomach injected with hot Gilson's fluid in order to fix the worms in position in the stomach. When the worms were counted on post mortem exami-

nation, a very unusual sex ratio was discovered. There were 1,474 males and 92 females, a finding that accounts for the terminal drop in the egg count without any sign of an increase in the number of red blood cells.

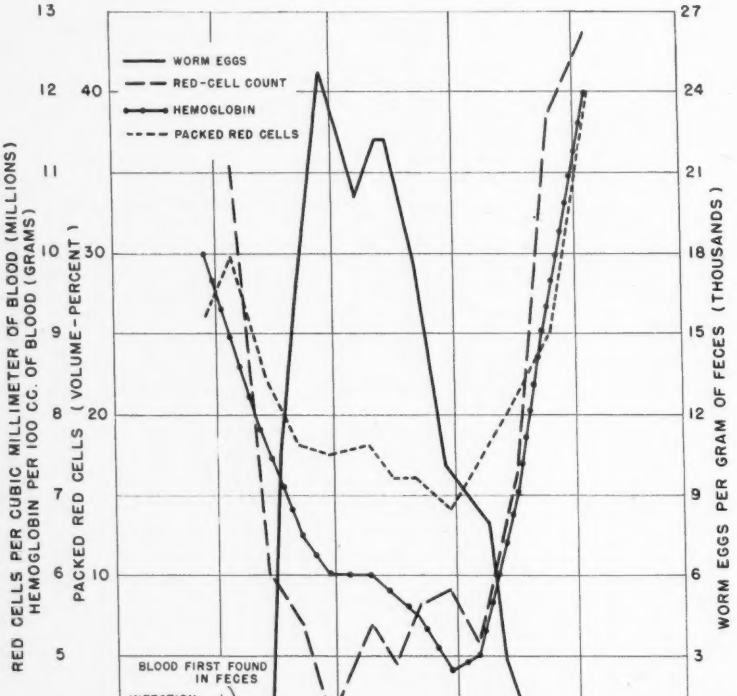

FIGURE 3.—Red cells and hemoglobin in the blood of lamb 9 correlated with the number of worm eggs per gram of feces passed after experimental infection with *Haemonchus contortus*.

Figures 5 and 6 present the results obtained for lambs 11 and 12 respectively. The blood pictures are similar to that of lamb 5 but, as already stated, no worm eggs were passed in the feces of either lamb 11 or 12. In both of the last-mentioned lambs, the number of red cells and the quantity of hemoglobin decreased to practically the same level at death.

According to figures 5 and 6, 1,492 and 2,380 cc. of blood were recovered from the feces of lambs 11 and 12, respectively, during the 10 days that the feces of these lambs were analyzed. Each of these lambs weighed about 25 pounds. According to Hodgson (*5*), who states that the weight in pounds multiplied by 38 gives the blood vol-

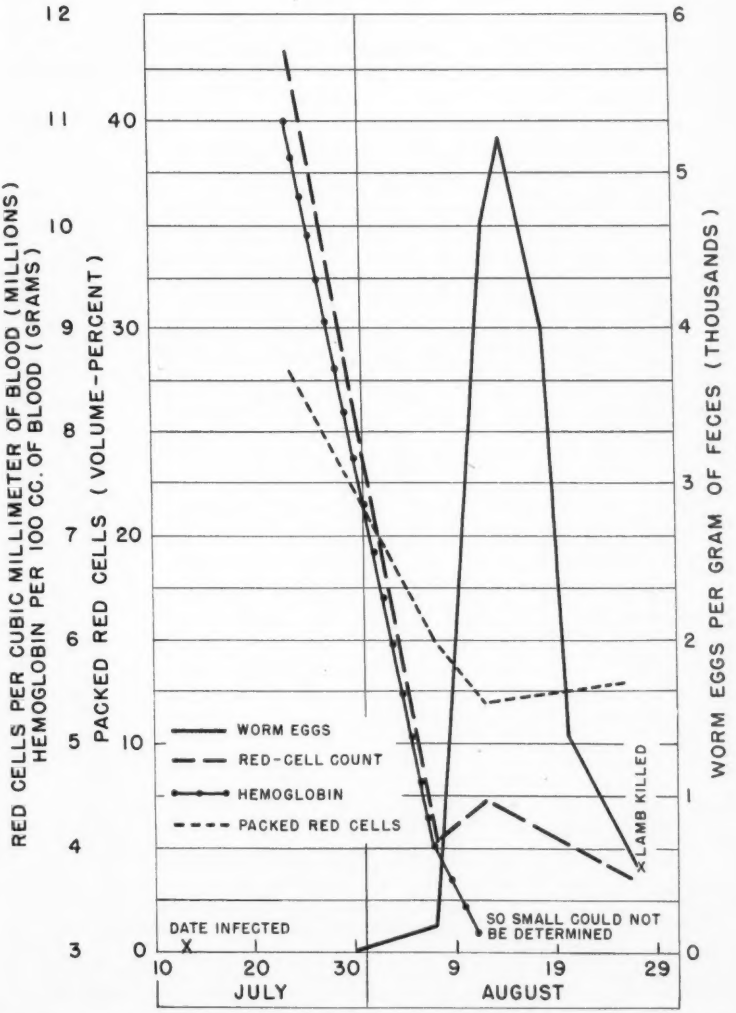

FIGURE 4.—Red cells and hemoglobin in the blood of lamb 5 correlated with the number of worm eggs per gram of feces passed during experimental infection with *Haemonchus contortus*.

ume, each animal normally had about 950 cc. of blood. Therefore, 1.57 and 2.50 times the total blood volume were recovered from the feces of lambs 11 and 12, respectively, during the entire period of infection. The difference in these figures may be due to the fact that the onset of hemorrhage in lamb 11 following the administration of the single large dose of larvae was more sudden and the loss of blood more rapid than in lamb 12, which received smaller doses of larvae over a

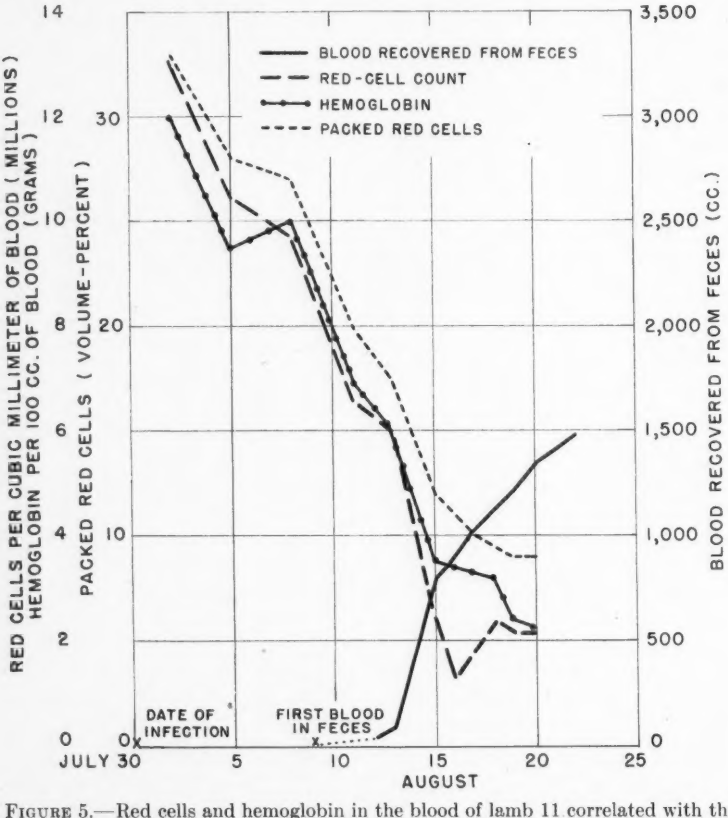

FIGURE 5.—Red cells and hemoglobin in the blood of lamb 11 correlated with the quantity of blood recovered from the feces passed after experimental infection with *Haemonchus contortus*.

period of time. In the second lamb the loss of blood was apparently less rapid at first. This circumstance permitted a greater restoration of the blood lost than was possible in the first lamb. The slower hemorrhage would therefore result in the loss of a greater volume of blood while producing the same degree of anemia as in lamb 11.

Although the quantitative relationship between the volume of blood recovered from the feces and the volume of blood lost by hemorrhage into the abomasum is at present unknown, since losses from digestion,

assimilation, and bacterial decomposition undoubtedly occur, such losses would tend to increase rather than to decrease the quantity of blood at the source of hemorrhage represented by a unit quantity of hemoglobin recovered from the feces. Therefore, any error involved in the determination due to this factor would not invalidate the conclusions drawn from the present analyses.

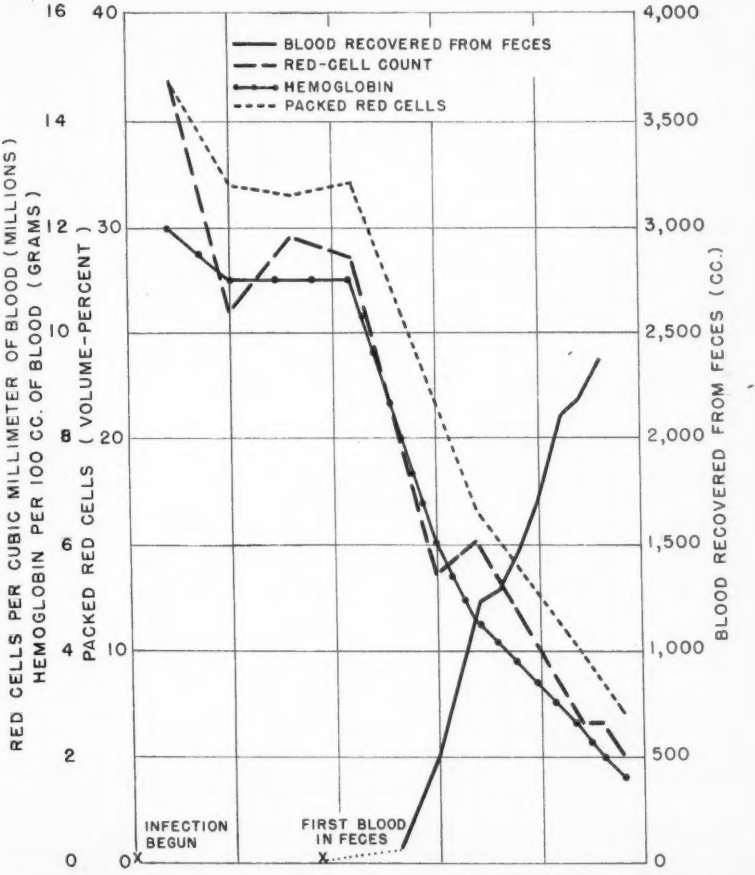

FIGURE 6.—Red cells and hemoglobin in the blood of lamb 12 correlated with the quantity of blood recovered from the feces passed during experimental infection with *Haemonchus contortus*.

As already stated, lamb 17, which was used as a control in this phase of the work, was bled daily, except Sundays, from the jugular vein. The data obtained are plotted in figure 7. As is shown in the figure, 3,560 cc. of blood were removed from this lamb during a period of 15 days. At the end of that time the animal had slightly more than 2 million red cells per cubic millimeter of blood, a stage of anemia

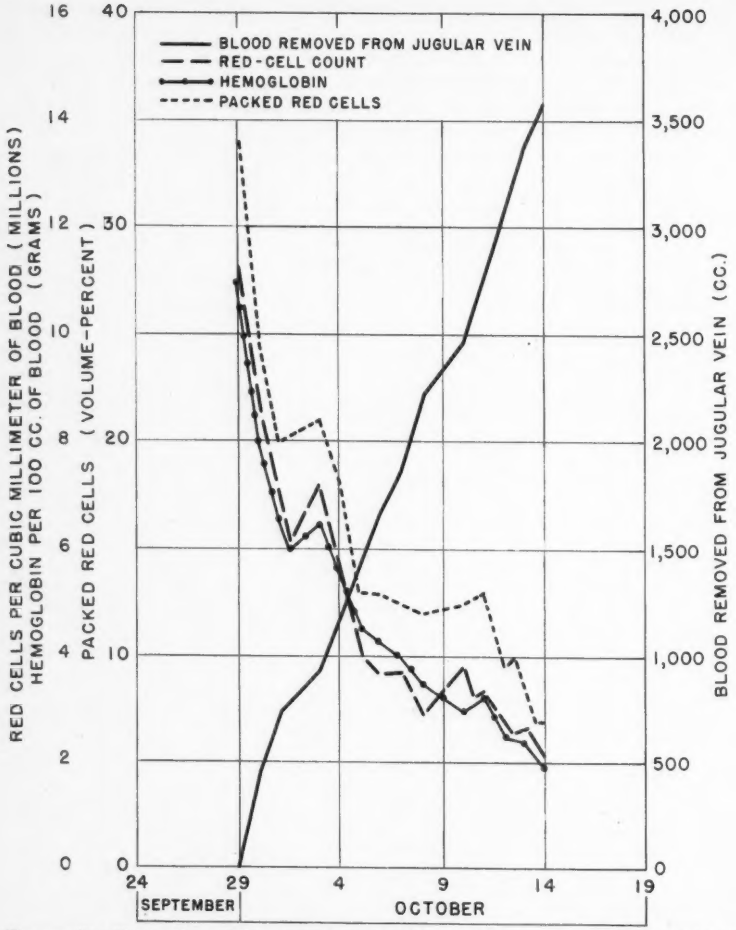

FIGURE 7.—Red cells and hemoglobin in the blood of lamb 17 correlated with the quantity of blood removed from the jugular vein.

comparable to that in lambs 11 and 12. Lamb 17 weighed 38 pounds, and its total blood volume was 1,444 cc., according to Hodgson (*5*). About 2.47 times its blood volume, therefore, was removed in producing this degree of anemia by the end of the 15-day period during which it was bled. When an allowance is made for the replacement of lost blood during the two Sundays that lamb 17 was not bled, the quantities of blood lost in the three lambs (Nos. 11, 12, and 17) agree sufficiently well to show that the anemia in stomach worm disease in sheep is definitely caused by hemorrhage.

CONTROL LAMBS

The average values for the number of red cells per cubic millimeter of blood, the quantity of hemoglobin per 100 cc., and the volume

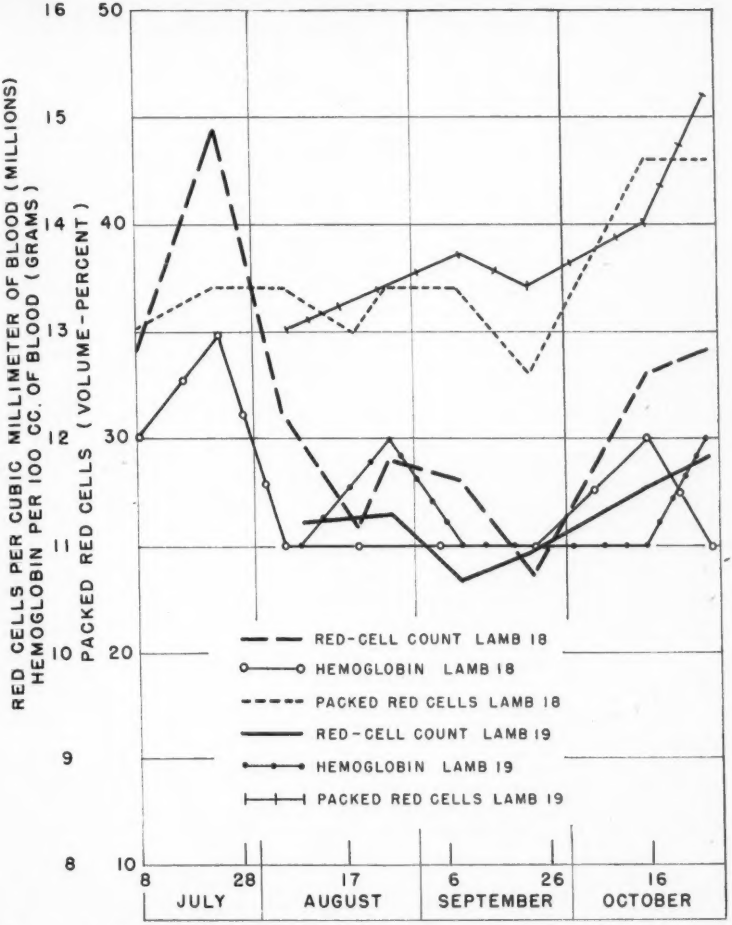

FIGURE 8.—Red cells and hemoglobin in the blood of control lambs 18 and 19.

percentage of packed red blood cells for the control lambs 18 and 19 are given in figure 8. These data show that the blood of the two lambs remained normal in these respects during the entire time that they were under observation.

RETICULOCYTE COUNTS

The infected lambs 9, 11, and 12 and the bled lamb 17 were considered to be typical of all the infected lambs in number of reticulocytes per 1,000 red cells. Therefore, these animals were selected for this phase of the study. The results are shown in table 2.

TABLE 2.—*Number of reticulocytes per 1,000 red cells in three lambs infected with Haemonchus contortus and in lamb 17, bled from the jugular vein*

Lamb 9		Lamb 11		Lamb 12		Lamb 17	
Date	Reticulocytes	Date	Reticulocytes	Date	Reticulocytes	Date	Reticulocytes
	Number		*Number*		*Number*		*Number*
Aug. 26	0	Aug. 2	0	Aug. 2	0	Sept. 29	0
Aug. 28	0	Aug. 8	0	Aug. 8	0	Sept. 30	0
Sept. 4	0	Aug. 11	0	Aug. 11	0	Oct. 1	0
Sept. 13	0	Aug. 13	0	Aug. 13	0	Oct. 3	15
Sept. 21	21	Aug. 17	0	Aug. 17	0	Oct. 4	44
Sept. 28	31	Aug. 18	83	Aug. 18	0	Oct. 5	41
Oct. 9	2	Aug. 19	160	Aug. 19	5	Oct. 6	55
Oct. 15	0	Aug. 20	80			Oct. 7	76
Oct. 21	11			Aug. 22	13	Oct. 8	80
Oct. 29	2			Aug. 23	23	Oct. 10	86
Nov. 5	11			Aug. 24	50	Oct. 11	100
Nov. 15	0					Oct. 12	95
Nov. 24	0					Oct. 13	129
Dec. 3	0					Oct. 14	75

As is shown in table 2, there was a great variation in the number of reticulocytes found in the blood of the four lambs at different times during the development of the anemia. This variation was apparently due to the individual reaction of each lamb to the suddenness and severity of the experimental infection. Since the number of reticulocytes in lamb 11 was even higher than that in lamb 17, there appeared to be no evidence that hematopoiesis was interfered with by toxins of any kind. No reticulocytes were found in the blood of the control lambs.

TOTAL AND DIFFERENTIAL WHITE-CELL COUNTS

The data on the total and differential white-cell counts for the experimental and control lambs are shown in table 3.

Statistical analysis of the data in table 3 indicated the following significant differences. The number of white cells per cubic millimeter of blood in each group of experimental lambs was greater than that of the controls, thus indicating that the same changes in the total white-cell count occurred when hemorrhage was the sole cause of the anemia as when it was brought about by the *H. contortus* infection. The lambs dying from infection and the bled lamb had higher percentages of polymorphonuclear cells than the controls. The first-mentioned group also had more polymorphonuclear cells than the lambs that recovered from the infection. The group that recovered, on the other hand, had a higher percentage of eosinophiles than the controls and fatal cases, a finding which indicated a positive correlation between eosinophiles and the development of resistance to the infection. The percentages of basophiles and monocytes were so small that no conclusions could be drawn from the observations. The differential counts of the lambs that died from the infection and those of the bled lamb were practically the same.

TABLE 3.—*Average total and differential white-cell counts of the lambs*

Lamb group	Lamb No.	Total white-cell counts made	Total white-cell counts per cubic millimeter of blood	Differential white-cell counts made	Lymphocytes	Polymorpho-nuclears	Eosinophiles	Basophiles	Monocytes	Smudges
		Number	*Thousands*	*Number*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Recovered from disease	3	15	7.81	14	75.64	22.40	1.37	0.44	0.00	0.15
	4	13	9.63	13	87.87	14.58	3.05	.32	0.	.27
	9	13	6.28	14	62.87	35.96	.69	.18	.11	0
	Average	.....	7.91	.....	75.16	24.55	1.67	.31	.04	.13
	Standard deviation	.....	±14.10	.....	±82.00	±84.86	±11.91	±4.23	±0.64	±1.93
Died from infection	5	5	9.36	5	63.30	36.50	.10	.10	0	0.0
	11	11	8.74	7	69.28	30.56	.14	.03	0	0
	12	10	10.69	9	69.56	29.85	.15	.06	0	.37
	Average	.....	9.61	.....	67.98	31.67	.13	.06	0	.16
	Standard deviation	.....	±11.05	.....	±47.21	±48.56	±1.15	±0.74	0	±2.27
Bled from jugular vein	17	14	8.09	14	69.15	30.54	.09	.00	.09	.07
	Standard deviation	.....	±4.70	.....	±25.32	±24.53	±0.57	0	±.81	±1.00
Controls	18	9	6.36	7	81.67	16.51	.41	.84	0	.54
	19	6	7.13	6	74.50	23.83	.42	.52	.23	.50
	Average	.....	6.67	.....	78.37	19.89	.41	.69	.11	.52
	Standard deviation	.....	±3.26	.....	±24.25	±23.91	±1.50	±3.26	±.69	±3.55

PATHOLOGICAL FINDINGS

Evidences of hemorrhage produced in the abomasum by *Haemonchus contortus* have been repeatedly observed by the author on lambs at autopsy. However, in order to clarify the pathological picture produced by this nematode and relate it to the life history of the worm, the following observations were made on lambs 13, 14, 15, and 16, which were given large doses of infective larvae and were then killed at different intervals after infection (table 1). Four days after infection the larvae were found between the papillae of the mucosa of the abomasum. There was no evidence of injury to the mucosa nor of hemorrhage, and the larvae had not attached themselves to the mucosa. Seven days after infection the surface of the mucosa of the abomasum was covered with blood coagula and mucus (pl. 1, *H*). The young, fourth-stage larvae were found under these coagula next to the mucosa but apparently were not attached to it. In 11 and 23 days after infection, the aspect of the stomach had changed little except that both the larvae and the coagula had increased in size and the quantity of blood within the stomach had increased markedly. Profuse hemorrhage was present in the abomasum of lamb 16.

Pathological observations also were made on lambs 11 and 12, which died as a result of infection, and on lamb 18, a control. Plate 1, *C*, shows lamb 12 a few minutes before death. This lamb, as contrasted with control lamb 18 (pl. 1, *A*,) was noticeably weak and unable to hold up its head or to stand firmly on its four feet. *F* shows the paleness of the white of the eye of lamb 12, caused by the severe anemia. A

normal eye is shown in *D*. *I* shows some of the viscera of lamb 12 at autopsy. In contrast to *G*, which shows the organs of control lamb 18, the viscera of lamb 12 have a bloodless appearance in spite of the fact that the lamb was not bled out prior to necropsy. All these illustrations show the severe anemia that is characteristic of *H. contortus* infection in lambs.

Plate 1, *E*, shows the abomasum of lamb 12 with many hundreds of young adult *Haemonchus contortus* attached to the mucosa in the pyloric region. This localization of the worms is unusual as they are usually found scattered over the entire mucosa.

Sections of the stomach, liver, heart, lung, kidney, and spleen of lambs 11 and 12 were sent to the Pathological Division of the Bureau of Animal Industry for histological examination. The report on these organs indicated some slight degenerative changes in the heart muscle, liver, and kidney, which may have occurred post mortem, and some pneumonic changes in the lungs, due to causes other than the nematode infection. The spleen and stomach, which are of particular interest in this instance, were described as follows:

> Spleen. There was comparatively little blood remaining in the splenic sinuses, and the rather limited quantity of blood throughout the splenic pulp, as compared with the normal, is indicative of anemia of this organ.
>
> Stomach. Sections from the stomach showed extensive sloughing of the mucosa, and masses of cellular debris could be seen deposited on the surface of the remaining intact mucosa. There was no congestion, and erythrocytes were present only in limited numbers. Lymphoid cells were present in rather large numbers and these cellular elements, together with limited numbers of plasma cells and eosinophiles, could be seen invading various portions of the mucosa. Slight fibroblastic proliferations were also noted in sections. The submucosa and muscularis showed evidence of edema. Sections of stomach worms were seen in close contact with the surface of the mucosa, and a number of the parasites had penetrated, or burrowed beneath the surface and were partly or completely embedded in the upper third of the mucosa. The stomach lesions [pl. 1, *B*] found in lambs 11 and 12, give evidence that these parasites are capable of causing considerable damage to the mucosa, particularly when present in large numbers. Such injury is doubtless sufficient to cause more or less continuous capillary hemorrhage, resulting eventually in extreme anemia and death of the animal.

These data, therefore, support the conclusion suggested by the data given previously in this paper that the anemia of *H. contortus* infection in sheep is due to hemorrhage.

DISCUSSION

The observations reported in the present paper on the hematology of *Haemonchus contortus* infection in sheep agree in general with the findings of other workers. The pathological changes found in the abomasum and associated with the infection were similar to those described by Veglia (*8*) and Fourie (*4*) except for the absence of the gross ulcerations described by the latter. The histological examination of the organs of lambs dying of the infection, however, did not disclose the peculiar crystals in the liver described by Fourie, but simply gave evidence of the injuries produced by the nematodes in the wall of the fourth stomach, of the capillary hemorrhage resulting therefrom, and of the extreme anemia.

The conclusions of other workers concerning the possible direct relation between the anemia associated with *H. contortus* infection in sheep and gastric hemorrhage have been given added validity by the

Pathological findings in some of the experimental lambs: *A*, Uninfected control lamb 18; *B*, section of stomach of lamb 12 showing destruction of the mucosa; *C*, lamb 12 shortly before death from stomach worm infection; *D*, eye of normal uninfected lamb; *E*, stomach of lamb 12 showing young adult of *Haemonchus contortus* (dark areas in lower right portion of photograph) attached to the pyloric region; *F*, eye of lamb 12, showing paleness of white; *G*, viscera of uninfected control lamb 18; *H*, stomach of lamb 14 killed 7 days after infection; *I*, viscera of lamb 12, bloodless in appearance in contrast with normal viscera.

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results of the analyses for blood in the feces of infected sheep, made during the present investigation. The conclusions drawn from this work were similar to those arrived at by Wells (*9*), who calculated that in dogs 1,000 hookworms could produce a loss to the host of 360 cc. of blood per day, a loss which, if continued, would result in a severe or fatal anemia. Foster and Landsberg (*3*) later came to the conclusion that the presence of a toxin was not necessary to account for the anemia of hookworm disease in dogs, since the anemia was like that associated with chronic hemorrhage.

SUMMARY

In a study involving *Haemonchus contortus* infection in sheep, 19 crossbred Hampshire-Southdown lambs 2 to 8 months old were used. The work was carried on at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md., in 1933 and from 1936 to 1938, inclusive.

From 2,000 to 181,000 infective larvae of *H. contortus* were administered to lambs in single and multiple doses. Although one dose of 35,000 larvae undoubtedly would have produced death in lamb 5 had this animal been allowed to die naturally, much larger total doses were not fatal when administered in small daily doses. This observation indicates the development of resistance to infection in the lambs receiving the multiple doses.

The data show that *H. contortus* produced a severe and sometimes fatal anemia in sheep. Blood appeared in the feces 6 to 10 days after infection. In the two cases which were fatal, blood was present in the feces until the death of the animals. In lambs recovering from the infection the anemia was negatively correlated with the number of worm eggs per gram of feces. In lambs dying before the worms matured anemia developed rapidly, but a positive diagnosis of the infection was impossible before autopsy owing to the absence of worm eggs in the feces. In two of the fatal cases, no worm eggs were passed in the feces, whereas one of the nonfatal cases had an egg count of 24,800 eggs per gram of feces. Accordingly, there was no correlation between the number of eggs per gram of feces and the fatality of the infection.

The quantities of blood lost during a period of 10 days in two of the lambs that died were 1,492 and 2,380 cc., or 1.57 and 2.50 times, respectively, the original quantity of blood in the lambs, as calculated from the body weight of the animals. These quantities compared favorably with the 3,560 cc. of blood taken from the jugular vein of a healthy lamb over a period of 15 days, until approximately the same degree of anemia had been produced. This volume of blood was about 2.5 times the original volume of blood in this lamb, as calculated from its body weight. That the anemia associated with *H. contortus* infection in sheep was due to gastric hemorrhage alone was further indicated by the normal icteric index, the presence of reticulocytes in the blood of the anemic lambs, and the gross pathology and histological findings.

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ARRANGEMENT OF THE TISSUES BY WHICH THE COW'S UDDER IS SUSPENDED [1]

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INTRODUCTION

The broken-down, pendulous udder is a defect that occurs frequently among high-producing dairy cows. There are a number of different types of broken-down udders. Some become detached from the body wall, so that the hand can be inserted between the abdomen and the upper surface of the front quarters. Some become elongated vertically like a sack and have a tendency to swing from side to side as the cow walks. In some the median support appears to undergo a relaxation, permitting the floor of the udder to sag and causing the teats to point outward from the sides of the udder. In others the rear attachment appears to become lax, allowing the udder to swing forward, and sometimes causing the rear quarters to occupy a position lower than the front quarters. These are the more common types of broken-down udders; there are many variations.

Generally, this breaking down is a condition that occurs with large, heavy udders. Usually it becomes progressively more pronounced with advance in age. Occasionally it occurs with udders of moderate size and may be pronounced early in life.

The breaking down of udders is frequently attributed to heavy milk production. The physiological function of milk secretion has been developed by breeding and improved nutrition until it has reached amazingly high levels. The range cow produces only enough milk to raise her calf. The good dairy cow produces in 1 year an amount of milk that is equivalent to 8 to 12 times her live weight. In some cases cows have produced an amount of milk in 1 year that was equivalent to 20 times their live weight. The combined weight of the udder and the milk that it contains is often very great.

The broken-down or pendulous udder is objectionable for a number of reasons. Such udders are difficult to milk; they are hard to keep clean; they interfere with the cow's locomotion; they are subject to friction, trauma, and injuries from sharp objects; and the teats are often injured as a result of being stepped on by the cow herself or by cows in adjoining stalls. Moreover, such udders are unsightly. Good-producing cows that have pendulous udders have much lower sale value than cows with well-attached udders, although little is known of the effect of this condition on the animal's producing ability. In view of the greater liability of the pendulous udder to injury such an udder would appear to be more subject to mastitis.

A more comprehensive knowledge of the supporting tissues of the udder may suggest methods of management that will be helpful in preventing broken-down udders; it may suggest points to be consid-

[1] Received for publication July 17, 1941.

ered in the selection of cows that are less likely to be susceptible to this trouble; or it may suggest points to be considered in the selection of animals for a breeding program having for its objective the fixing of an inheritance for strongly supported udders. With these facts in mind a study was undertaken to determine the nature of the structures that support and maintain the udder in well-balanced suspension. The results of the study are presented in this paper.

REVIEW OF LITERATURE

A recent tabulation of the weights (after milking) of excised udders of cows that were slaughtered by the Bureau of Dairy Industry, in connection with its studies of conformation and anatomy in relation to producing capacity at Beltsville, Md., gives some idea of the size of the udder of high-producing dairy cows.[2] Data were available for 50 lactating Holstein-Friesian cows 4 years of age or over. On an average the weight of the udder declined with advance in the stage of lactation. The udders of 17 cows that had been milked 3 months or less in the current lactation at the time of slaughter averaged 72.98 pounds; those of 25 that had lactated 6 months or less averaged 65.07 pounds; those of 35 that had been milked 9 months or less averaged 58.08 pounds; those of 41 that had been in milk for 12 months or less averaged 55.89 pounds; and the entire group of 50 udders had an average empty weight of 52.47 pounds. Among these there was 1 that weighed 165.65 pounds, which was approximately 11 percent of the live weight of the cow; and 2 others that weighed 138.20 and 101.85 pounds, respectively.

The results of a number of investigations indicate the weights of blood and milk that the udder may carry.

Swett, Miller, and Graves (*8*)[3] found that a large proportion of the milk obtained at a milking is stored in the udder before the milking process is commenced. Thus a heavy-milking cow producing 100 pounds of milk on twice-a-day milking might have 50 pounds or more of milk in the udder before each milking.

Shaw and Petersen (*6*) found that for each pound of milk secreted, nearly 400 pounds of blood passes through the udder. This means that for a cow producing 100 pounds of milk daily, the blood passing through the udder in that length of time would amount to approximately 40,000 pounds. The total quantity of blood in the body of a lactating cow has been determined (*2, pp. 54–55*) to be approximately 8 percent of the cow's live weight. This means that the bodies of few cows would contain more than 150 pounds of blood. Presumably only a small proportion of the total amount of blood would be present in the udder at any time. According to Kay (*5*) the udder in full lactation takes about one-quarter of the heart's output of arterial blood.

Thus the total weight of the milk and blood in the udder at any time might easily be 50 to 60 pounds, but probably would not exceed 75 pounds, except in unusual cases. This would mean that the total weight of a large, heavy-producing udder, together with its contents, might vary between limits of approximately 100 to 250 pounds. In view of the great weight of the functioning udder and its contents it is

[2] Unpublished data.
[3] Italic numbers in parentheses refer to Literature Cited, p. 43.

not surprising that in many cases the anatomical structures by which the udder is suspended are inadequate to support it in its proper position.

There are some indications that the tendency for the udder to break away from its attachments is inherited. It appears to occur with high frequency among the daughters of certain sires.

The weakness may result from poor tonus in the supporting structures. According to Hammond (*4*) the formation of pendulous udders is the result of excessive and continued internal pressure produced by the accumulated milk in the udder.

Some believe that a weakness in the support of the udder causes faulty circulation which tends to produce stasis, edema, fibrosis, and an increase in the size and weight of the udder. This in turn would further stimulate the breaking-down process and make the abnormalities resulting from faulty circulation even more pronounced.

Sisson (*7, p. 620*) describes the suspensory apparatus as follows:

> The mammary glands, normally two in number, are popularly termed the udder. They are very much larger than in the mare, and the body of each is somewhat ellipsoidal in form, but flattened transversely. The base of each gland is slightly concave and slopes obliquely downward and forward in adaptation to the abdominal wall, to which it is attached by means of a well-developed suspensory apparatus (Lig. suspensorium mammaricum) which extends backward and is attached to the symphysis pelvis by means of the strong plate of tendinous tissue (Tendo subpelvina). This plate of tissue attaches the prepubic tendon to the ventral part of the symphysis. The suspensory apparatus consists essentially of four sheets of tissue two of which are well developed and median in position and are chiefly yellow elastic tissue; the two glands are separated by this double septum which attaches to the medial flat surface of each gland. The lateral sheets (containing less elastic tissue), arise from the subpelvic tendon posterior to the udder; on reaching the abdominal floor they diverge and pass laterally to the external inguinal ring. They extend downward over the udder and divide into superficial and deep layers: the superficial layer attaches to the skin where it reflects off the udder to the medial face of the thigh, and the deep layer is thicker and attaches to the convex lateral surface of the udder by numerous lamellae which pass into the gland. It is in relation posteriorly to the large supramammary lymph-glands and a quantity of fat.

Bitting (*1*) was one of the first in this country to publish results of anatomical studies of the suspensory apparatus of the cow's udder. His description was general and included nothing of importance that is not covered by that of Sisson (*7*), except that he emphasized the importance of the strength of the abdominal wall as a factor in determining the shape and apparent size of the udder. He states (*1, p. 39*):

> In a cow with loose abdominal walls, dropping directly down from the pubis, thus forcing the udder downward and backward, the organ will appear to be much larger than in one in which the walls are stronger. This sometimes accounts for the apparently sudden development of a good udder after the second or third calf. The muscles of the abdomen become more relaxed and the udder becomes more pendulous.

An interesting and valuable contribution to the subject was made by Emmerson[4] who dissected several cow udders and described and illustrated many of the supporting structures. Emmerson elaborated considerably with regard to the lamellae (plates) which are given off by the deep faces of the lateral sheets, and penetrate the glandular tissue in a medioventral (inward and downward) direction to become incorporated in the insterstitial framework of

[4] EMMERSON, M. A. STUDIES OF THE MACROSCOPIC ANATOMY OF THE BOVINE UDDER AND TEAT. 1928. [Unpublished thesis. Copy on file in library of Iowa State Col. of Agr. and Mech. Arts, Ames.]

the udder. He also pointed out that the lateral suspensory ligamentous sheets continue to the ventral (lower) border of the gland at which point the lamellae anastomose and become continuous with those of the sheets of elastic tissue which make up the median septum, thus forming a sling or cradlelike structure in which the udder is held. Unfortunately the results of Emmerson's work are not available in published form. However, his work has been quoted rather freely by Turner[5] and by Espe (*3, pp. 9–10*).

There is no question as to the accuracy of Sisson's brief description of the udder's suspensory structures, or of Emmerson's elaboration concerning them. To the anatomist they are, without doubt, understandable and adequate. However, an amplified description expressed in simpler terminology and liberally illustrated should enable the research worker, the cattle judge, the dairy farmer, and all others interested in dairy cattle problems, to visualize and understand more clearly the nature of mammary-gland suspension in the cow.

In evaluating the udder capacity of living cows it would be desirable to be able to calculate the volume of the udder. To do this it is necessary to have a knowedge of the shape of the dorsal (upper) surface of the udder, and of the curvature and degree of slope of that part of the abdominal wall to which the fore udder is attached. It has been pointed out by the authors (see *9, p. 9*) that the rear and front quarters of the udder generally produce in the ratio of 3 to 2 (58.2 percent from the two rear quarters and 41.8 percent from the two front quarters), and that a similar ratio existed between the depth of the rear and front quarters of the excised udders studied (10.29 inches for the rear and 6.38 inches for the front). It is well known, of course, that the abdominal wall is lower at the anterior attachment of the udder than at more posterior points and that it curves upward toward the rear. But the shape of the curve of the abdominal wall from front to rear, or from side to side, has not been readily or accurately measured in the living cow. Neither has the outline of the dorsal surface of the udder been accurately ascertained.

PLAN OF STUDY

In order to obtain additional information that might throw light on the problems of the broken-down or pendulous udder in heavy-producing cows, and also to obtain data that might make it possible to calculate the volume of udder of the living cow, a plan was outlined for studying in detail the structures suspending the udder of a cow that was known to be a good producer. Because of the desirability of obtaining photographs of dissections of these structures in their normal position, the plan called for the work being done with the animal in a standing position. This was accomplished by following the methods that are later described.

HISTORY AND CHARACTERISTICS OF COW USED

Holstein-Friesian cow No. 1216 was selected for this study. She was 5 years 10 months of age and had been in milk for 7 months of the fourth lactation period when slaughtered. Her live weight, 4 months before slaughter, was 1,320 pounds. Her production record at 3 years 2 months of age was 14,734 pounds of milk and 540 pounds

[5] TURNER, CHAS. W. THE COMPARATIVE ANATOMY OF THE MAMMARY GLANDS WITH SPECIAL REFERENCE TO THE UDDER OF CATTLE. 378 pp., illus. Columbia, Mo. 1939. [Mimeographed.] See pp. 31-37.

of butterfat (equivalent to 17,533 pounds of milk and 643 pounds of butterfat at maturity).

The mammary-gland development of cow No. 1216 was slightly retarded until she was 2 months of age. From 3 to 5 months of age it was normal for the breed, and from 6 to 18 months it was definitely superior to the breed average. Up to 4 months of age there was a tendency for the attachments of the gland tissue to the body to be inferior. Subsequently, up to 18 months, the attachments were superior to the average. However, the attachment of the udder of this animal, both as a heifer and as a lactating cow, was poorer at the rear than at the front at nearly every examination. This was particularly noteworthy at the last examination, 3 days before slaughter, when she was given grades of 5 and 3+ respectively for front and rear attachments of gland tissue, when graded on a 9-point basis, in which 9 is excellent, 5 average, and 1 extremely poor. The gland tissue hung low in the rear quarters before slaughter and the separation between right and left halves was marked. During lactating life the udder was of fair shape and medium or above in size. The weight of the udder was not obtainable because of the method employed in slaughtering the cow and in preparing her for anatomical study. The

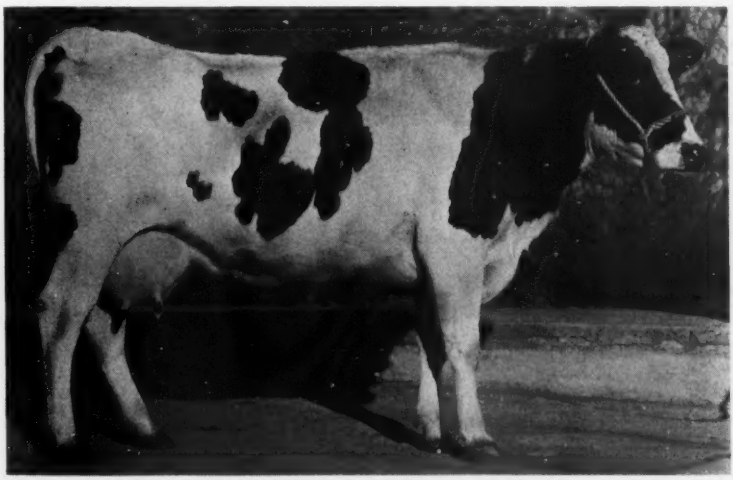

FIGURE 1.—Cow No. 1216, as she appeared about 1 month before she was killed.

udder was of medium quality and not excessively fibrous or meaty. It was somewhat inclined to be pendulous although this condition was not extreme.

Strip-cup examinations of the milk showed that this cow had had numerous flare-ups of mastitis of varying severity but of short duration. Mastitis was noted in all four lactation periods except the second. During both the first and third lactation periods all the quarters of the udder were affected at one time or another. Swelling and edema were noted prior to first calving, but neither was extreme. All of the edema had disappeared 15 days after calving and nearly all of the swelling was gone at the end of the first month. It is considered

improbable that either the mastitis or the swelling was of sufficient severity or duration to affect the suspensory structures or the attachments of the udder.

PROCEDURE FOLLOWED

The method generally employed in preparing large animals for dissection was used in this study. On the morning of January 25, 1939, the cow was rendered insensible by the injection of chloral hydrate and the subsequent administration of chloroform. She was then bled from an incision in the right carotid artery until heart action ceased, after which some residual blood was drawn by vacuum. The carotid artery and other severed blood vessels were tied off, and a glass tube, connected by a rubber hose to a tank located at an elevation some 12 or 14 feet above the cow, was inserted in the jugular vein and tied securely in position. By this means the filling of the circulatory system of the cow was commenced. A formalin solution of approximately 10-percent strength was used to preserve and harden the tissues. Meanwhile a heavy hook was inserted beneath the sacrum, and two smaller ones were inserted on opposite sides of the skull somewhat below and slightly posterior to the eyes (beneath the cheek bones or zygomatic processes of the malar bones). By means of ropes attached to these hooks the cow was hoisted into a standing position. An additional support in the form of a timber placed crosswise under the floor of the chest aided in holding the midportion of the cow at the proper height. A stoneboat was placed under the cow, the feet and legs of the cow were pulled into positions as nearly normal as possible, and each hoof was nailed to the stoneboat.

With the cow in this position, which was essentially normal except for the outstretched head, the filling of the circulatory system was continued. Within the first 3 hours about 30 gallons of formalin flowed into the jugular vein, and an additional 10 gallons was nearly exhausted some 3 hours later. During the course of the filling the udder became distended and many of the subcutaneous veins on the legs and under part of the body stood out prominently. Residual milk in the udder commenced to flow from the teats, and teat plugs were inserted. Incisions made for the supporting hooks were packed and sewed together to prevent escape of the formalin, and very little was lost. Some formalin was poured into the nostril to prevent or minimize fermentation in the rumen. Measurements of the circumference of the paunch on January 26 and 27 were 248 cm. and 247 cm., respectively. This lack of increase indicated that excessive fermentation had not occurred.

On January 27 a photograph was made showing the embalmed cow with all supports in place. The supporting apparatus was removed and the cow was again photographed. There was no significant change in the position of the cow, which showed that the tissues were adequately preserved and hardened to provide ample rigidity. The supporting equipment was replaced, however, to keep the embalmed body in an unchanged position until dissection was made. Figure 1 shows the appearance of the living cow about 1 month before she was killed. Figure 2, *A*, shows the embalmed, hardened cadaver standing, with the supporting mechanism and injection tube in position; and figure 2, *B*, shows the cadaver in the same position unsupported.

Mention has been made of the comparatively poor rear attachment of the udder which occurred in this animal as a calf and during lac-

tating life, and which was particularly noteworthy at an examination about a month before she was slaughtered. Figure 3, *A*, shows a rear view of the udder of the living cow, and figure 3, *B*, shows its appearance after she had been embalmed and hardened in situ.

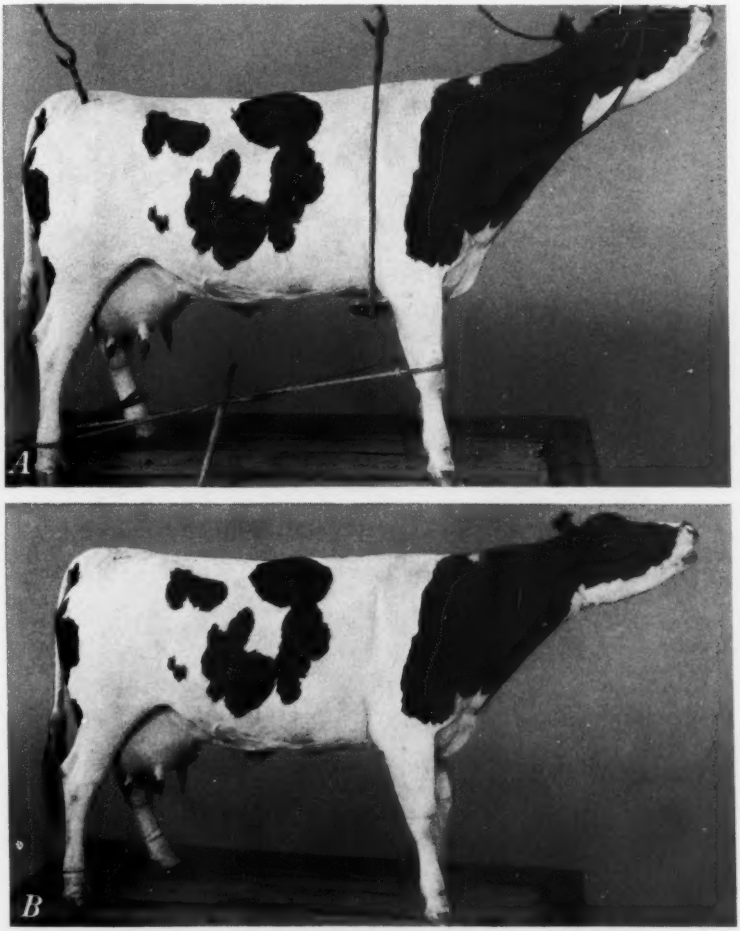

FIGURE 2.—The embalmed cadaver: *A*, With supporting equipment and injecting tube in position; *B*, with supporting equipment and tube removed.

Dissection was commenced on January 30. Photographs were taken as the work progressed and as one after another of the tissues by which the udder was suspended were uncovered. The dissecting and photographing work was carried on with the embalmed cow standing as shown in figure 2, *A*, with the hooks attached and the hoisting apparatus taut to prevent a change of position.

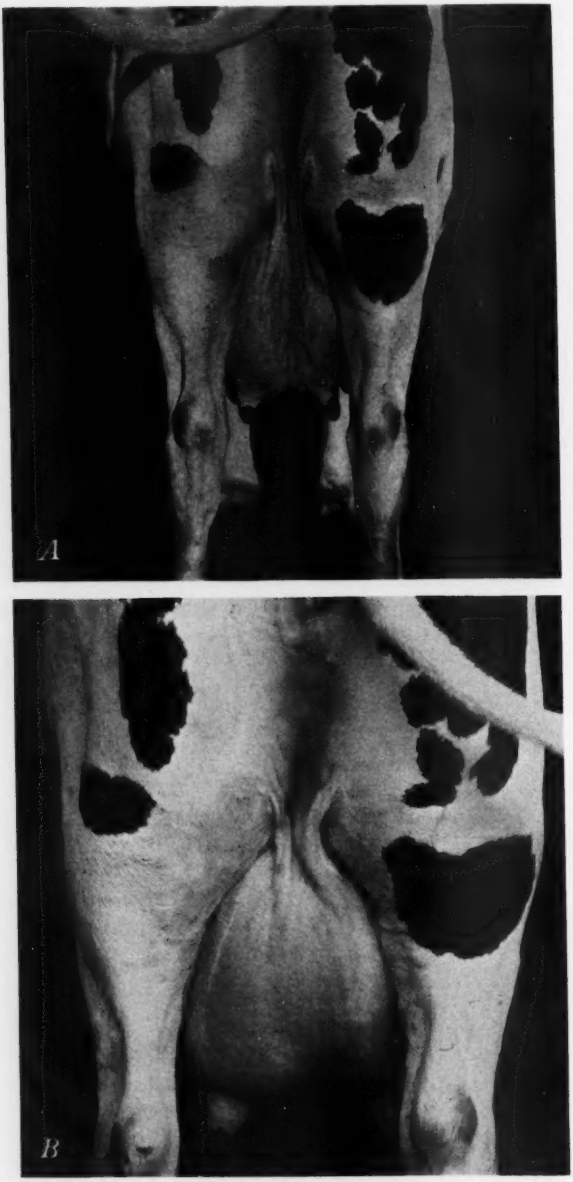

FIGURE 3.—*A*, Rear view of the udder before the death of the cow; *B*, its appearance after being embalmed.

PRESENTATION AND DISCUSSION OF RESULTS

At this point it may be helpful to list and number in the order found the principal parts of the suspensory apparatus of the udder, in order that they may be identified more easily by referring to the tissue number in the discussion of the step-by-step dissections by which they were brought to light. The principal parts of the suspensory apparatus are as follows:

Tissue No. 1.—The skin, which serves in a minor capacity to suspend and stabilize the udder.

Tissue No. 2.—The fine areolar, subcutaneous tissue (superficial fascia), which serves as an attachment between the skin and the underlying tissues.

Tissue No. 3.—The cordlike (coarse areolar) tissue, which forms a loose bond between the dorsal (upper) surface of the front quarters of the udder and the abdominal wall.

Tissue No. 4.—The pair of superficial layers of the lateral sheets of partly elastic tissue (lateral suspensory ligaments), which arise from the subpelvic tendon, extend downward and forward over the udder, and reflect off the udder to the medial (inner) face of the thigh.

Tissue No. 5.—The pair of deep, somewhat thicker layers of the lateral sheets, which have the same origin, extend downward over the udder and virtually envelop it, but which, unlike the superficial layers, attach to the convex lateral surfaces of the udder by numerous lamellae (plates) which pass into the gland and become continuous with the interstitial framework of the udder.

Tissue No. 6.—The subpelvic tendon. The tendon itself, strictly speaking, is not a part of the suspensory apparatus of the udder. Nevertheless it plays an important role as it gives rise to both the superficial and deep layers of lateral sheets described above.

Tissue No. 7.—The two adjacent sheets of heavy yellow elastic tissue, median in position, which arise from the abdominal wall, and attach to the medial flat surfaces of the two glands to form a double septum between them (median suspensory ligament).

A wide divergence of opinion exists with regard to the relative importance of these different parts of the suspensory apparatus. Emmerson [6] indicated that the median septum (tissue No. 7) is considered by most authors as the most important, but that his own observations led him to conclude that the less elastic lateral suspensory sheets (tissues No. 4 and No. 5) are the chief means of support and that the median septum merely holds the udder in close proximity to the posterior (rear) abdominal wall. In this connection he explained that the greater elasticity of the sheets comprising the median septum permits a lowering of the udder and an outward protrusion of the teats when the udder is distended with milk. He attached little importance to the fascia or areolar tissue (tissues No. 2 and No. 3) and indicated that the chief functions of the skin are to assist in the support of the fore part of the udder and to prevent undue pendulum movement.

An incision was made through the skin and subcutaneous tissue along the right flank and forward on the abdominal wall to a point somewhat anterior to the front attachment of the udder. In this manner an area of skin was dropped down exposing about half of the lateral surface of the udder and some of the abdominal wall. This showed a layer of fine areolar tissue (tissue No. 2) by means of which the udder is loosely attached to the skin. It is by means of such tissue that the skin assists in supporting and stabilizing the udder. Figure 4, *A*, shows the location and the appearance of the fine areolar tissue along the line *a*—*a*, where the skin is partly separated

[6] See reference cited in footnote 4.

from the underlying tissues. Areolar tissue of this type supposedly provides also the chief means of attachment between the hide in other parts of the body and the underlying structures. It is obvious from the well-known flexibility of the skin that this does not give a rigid attachment but permits considerable laxity and motility.

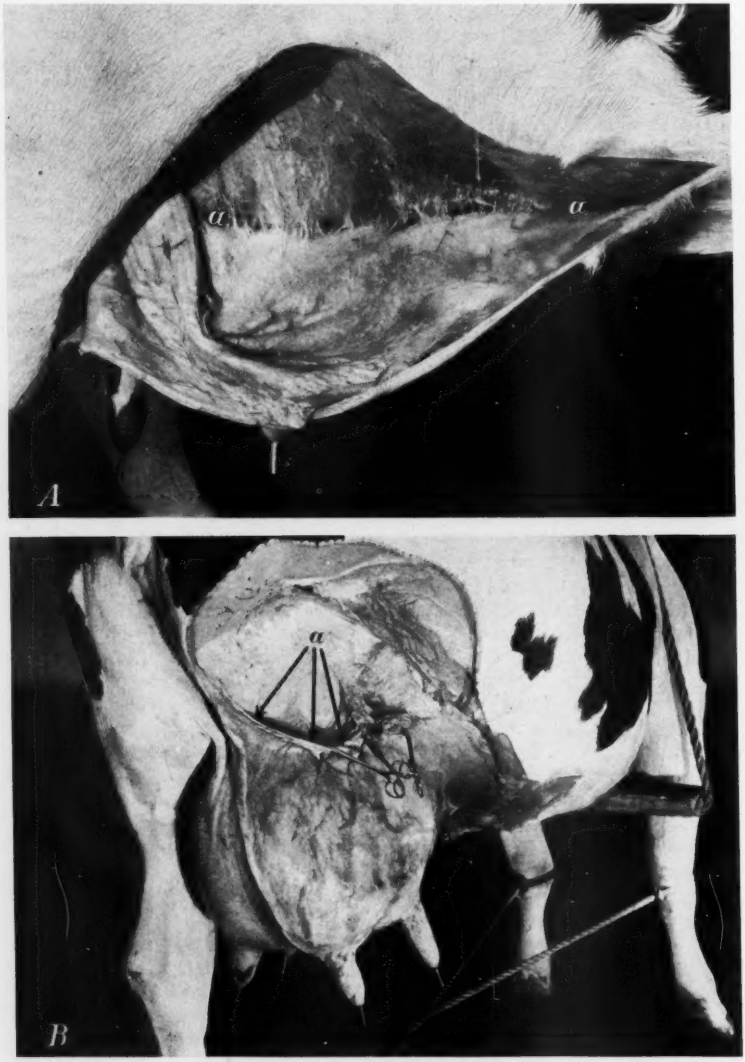

FIGURE 4.—*A*, Location and appearance of the subcutaneous fine areolar tissue (tissue No. 2) where the skin is folded down along the line *a*—*a*; *B*, portion of a partly enveloping sheet of fibrous tissue (tissue No. 4) which originally had been attached to the muscular tissues of the thigh. The severed edge is shown along the line indicated by *a*. Right hind leg removed.

The right hind leg was next removed at a point just below the hip joint, together with an area of skin extending from the tips of the teats upward to the level of the hip joint (thurl) and forward to a point somewhat anterior to the front attachment of the udder. This revealed more fine areolar tissue (tissue No. 2) and a sheet of fibrous partly elastic tissue which was still attached to the abdominal wall and which originally had been attached to the muscular tissues of the thigh. A portion of this sheet of tissue (tissue No. 4) was severed along the posterior dorsal (rear upper) surface of the udder, and is shown stretched out laterally by means of forceps attached to a cord

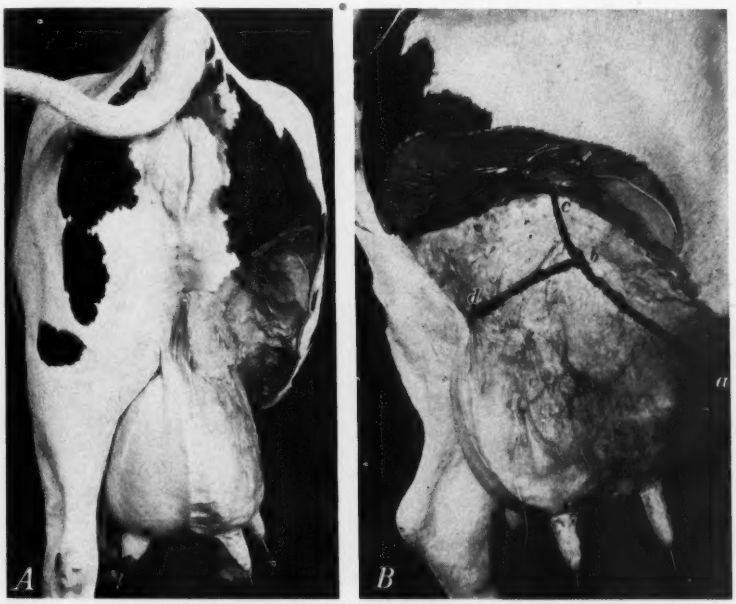

FIGURE 5.—*A*, Posterior view of the udder showing how it narrows dorsally to accommodate the thigh; *B*, lateral view with the upper boundary of the gland tissue outlined with a stain. Line *a*—*b*—*c* shows the line of the abdominal wall; *b*—*d*, the upper boundary of the udder. Note how the upper boundary of the udder follows the contour of the abdominal wall to a point approximately below the brim of the pelvis and then inclines downward toward the rear.

(fig. 4, *B*). It will be noted that this layer of tissue is fairly close to the median line at the rear and that it flares out toward the front. It partly envelopes the udder and serves as an important unit of its suspensory apparatus. The line on which the separation was made is indicated by *a*.

A posterior (rear) view of the udder at the same stage of dissection (fig. 5, *A*) shows the contour of the udder and indicates how markedly it narrows toward its dorsal (upper) extremity, especially at the rear, to provide ample space for the thigh. A lateral (side) view, also at the same stage of dissection, shows the approximate upper boundary of the gland tissue which has been outlined with a staining material (fig. 5, *B*). It will be noted that the upper boundary follows the

contour of the abdominal wall to a point almost directly below the brim of the pelvis, which is very close to a vertical plane passing through the two hip joints, after which, in this case at least, it inclines downward toward the rear. It is likely that in cows having a firm rear attachment of the udder, the upper surface of the gland tissue posterior to the brim of the pelvis may continue in a more nearly horizontal plane.

Figure 6, *A*, shows a posterior view of the udder after the removal of both hind legs. The posterior median support consisting of tissues

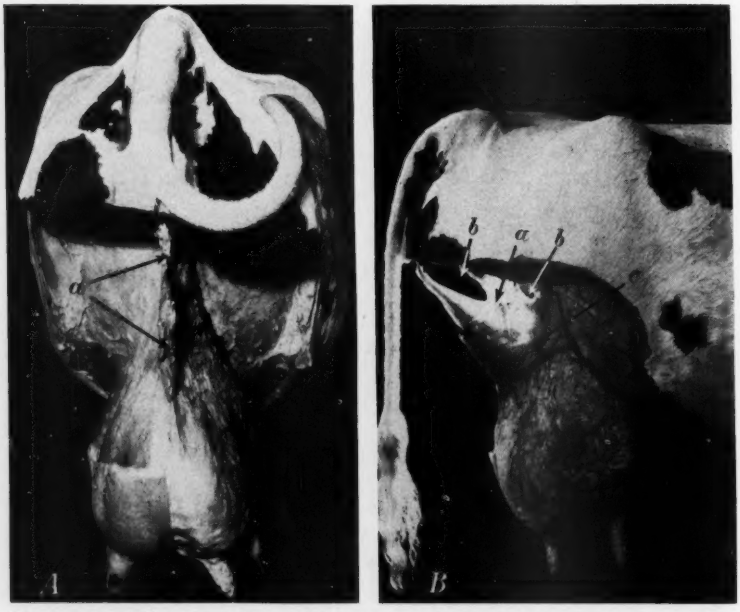

FIGURE 6.—*A*, Posterior view of the udder showing posterior median support at *a*, and indicating the contour of the udder, the angle of abdominal inclination, and the relatively small portion of the abdominal wall covered by the udder; *B*, lateral view showing the translucent subpelvic tendon (*a*) (tissue No. 6) which connects the lateral sheets of connective tissue (tissues No. 4 and No. 5) that are attached to the udder at *c* with the ventral surface of the pelvis. Open spaces in the subpelvic tendon are shown at *b*.

attached to the subpelvic tendon (tissue No. 6) is indicated by *a*. This view also gives a good idea of the contour of the udder itself, the angle of abdominal inclination, and the relatively small portion of the abdominal wall covered by the udder.

A better idea of the nature of the posterior median support of the udder is obtainable from the illustration in figure 6, *B*. This side view shows a closer dissection of the strong subpelvic tendon (tissue No. 6), which connects the lateral sheets of connective tissue that are directly attached to the udder (tissues No. 4 and No. 5) with the ventral (lower) surface of the pelvis (symphysis pelvis). This view, which was illuminated from the opposite side when photographed, shows how the subpelvic tendon becomes increasingly translucent (semi-

transparent) as it changes in structure from the mixture of fibrous and tendinous tissues at the udder, to a thin tendinous sheet where it attaches to the pelvic bone. The subpelvic tendon is not continuous but consists of a number of separated points of attachment along the ventral (lower) ridge of the pelvis. In figure 6, *B*, *a* indicates the translucent subpelvic tendon (tissue No. 6), *b* and *b* indicate the interstices (open spaces) in the tendon, and *c* shows the point where the lateral sheets of the udder's suspensory apparatus (tissues No. 4 and No. 5) arise from the subpelvic tendon.

Despite the inferior rear attachment of this udder its position was not so far forward as to appear unusual in the living cow. However, when the dissection had progressed to the stage illustrated in figure 6, *B*, the udder seemed to be located surprisingly far to the front although actually its position was essentially unchanged.

For purposes of record a number of measurements were made to establish more definitely the size of the udder, the extent of its attachment to the abdominal wall, and its position with reference to other points in the pelvic region. The following are some of the measurements obtained:

Measurement	*Centimeters (Inches)*	
(1) Distance from a perpendicular through the posterior point of the pinbone (ischium) to a vertical, transverse plane through the—		
(a) Posterior (rear) extremity of udder (right side)	22.0	(8.66)
(b) Most posterior (rear) extremity of abdominal wall (its junction with the brim of the pelvis)	38.5	(15.16)
(c) Anterior (front) extremity of udder (right side)	61.0	(24.02)
(d) Calculated length of udder	39.0	(15.35)
(2) Greatest width of udder at a vertical plane through rear teats	29.5	(11.61)
(3) Greatest width of udder at a vertical plane through front teats	32.75	(12.89)
(4) Maximum width of attachment to the abdominal wall	24.75	(9.74)
(5) Width of abdominal attachment at a vertical plane through front teats	20.75	(8.17)
(6) Width of abdominal attachment at a vertical plane through rear teats. (See dorsal boundary line in fig. 7, *B*)	8.50	(3.35)

Figure 7, *A*, shows a view in which the deep lateral layer of the suspensory apparatus (tissue No. 5) has been partly separated from the abdominal wall to show a layer of cordlike tissue lying between the dorsal (upper) surface of the udder and the abdominal wall. This tissue, which apparently provides the means by which most of the dorsal surface of the udder is attached to the abdominal wall, is areolar in type, very coarse, and loosely bound together. Presumably, in the event of excessive udder weight, or as a result of an inherited weakness or other causes, this tissue might give way and permit a separation between the udder and the abdominal wall with a characteristic breaking away of the front attachment of the udder. Emmerson's comment regarding the relative lack of importance of these tissues is worthy of note. Figure 7, *A*, *a* shows the loose cordlike tissue (tissue No. 3) and *b* the partly severed deep lateral layer of the enveloping suspensory tissue (tissue No. 5).

The completely severed deep lateral layer of the suspensory tissues which attaches to the convex lateral surface of the udder and virtually envelops it (tissue No. 5) is shown in figure 7, *B*. A portion of this layer below the horizontal incision was dissected away from the udder and folded downward along the line *b*—*b* (fig. 7, *B*) in such

FIGURE 7.—*A*, The deep lateral layer of enveloping suspensory tissue (tissue No. 5) partly separated from the abdominal wall at *b* to show cordlike tissue (*a*) (tissue No. 3) which serves to hold the udder in contact with the abdominal wall; *B*, the deep lateral layer (tissue No. 5) severed at *a* and folded back along the line *b*—*b*.

a manner as to reveal the two edges, indicated by *a*, which were formed by the incision. As shown in the illustration, this layer consisted of a clearly defined sheet of tough tissue which became continuous, ventrally (below), with the udder. The cordlike tissue along the abdominal wall (tissue No. 3) is shown here also, but less distinctly than in figure 7, *A*. It is conceivable that, in cases where the loose, cordlike tissue gives way, the enveloping layer of tissue and the median elastic support (tissue No. 7) may stretch sufficiently to permit a lowering of the udder—especially in front—which would thereby become pendulous.

The deep lateral layer (tissue No. 5) which previously had been severed (fig. 7, *B*) was separated from the udder to a lower level and folded downward along the line *a–a*, shown in figure 8, *A*. Figure 8, *A*, also shows how the dissection on the right side was carried deeper with the removal of a considerable quantity of fat and connective tissue, bringing to light the small areas of parenchymatous (secretory) tissue shown at *b*; the branch of one of the two yellow elastic supporting sheets of tissue (tissue No. 7) shown at *c*, which form the median septum of the udder; one of the large arteries where it enters the udder as shown at *d*; and a large vein on the dorsal (upper) surface of the udder at the point where it emerges from the udder as shown at *e*. The subpelvic tendon (tissue No. 6) is still attached (fig. 8, *A*, *f*).

Figure 8, *B*, shows the udder from the left at essentially the same stage of dissection as in figure 8, *A*, except that more of the deep, lateral enveloping layer of suspensory tissue (tissue No. 5) has been removed from the anterior (front) part of the udder, whereas at the rear of the udder a section of it (fig. 8, *B*, *a*) is still intact and attached both to the mammary gland and to the subpelvic tendon. A large vein extending along the abdominal wall at the upper surface of the udder is clearly visible (fig. 8, *B*, *b*).

It seems appropriate at this point to give attention to a matter that was not satisfactorily brought out in the gross dissection of the udder used in this study. Sisson (*7*), Emmerson,[7] Turner,[8] and Espe (*3*, *pp. 9–10*) mentioned the lamellae (plates) that are given off by the deep faces of the deep lateral sheets (tissue No. 5) which penetrate the udder and join with its intraglandular interstitial framework. A histological study of sections including the lateral sheets and the glandular tissues immediately beneath shows the manner in which the lamellae branch off and extend downward and inward to become continuous with the intraglandular tissues (pl. 1, *A*). This study brought out the fact that the sheets comprising the median septum (tissue No. 7) also give rise to lamellae which penetrate the glandular tissue in a similar manner (pl. 1, *B*).[9]

It is more difficult to determine to what extent the lateral and median sheets fuse at the ventral (lower) border of the gland to form a sling or cradlelike structure for its support. This is because of the laminated nature of both the lateral and median sheets and the fact that numerous branches are given off. A section through an udder that was included in routine studies of the Bureau of Dairy Industry shows a band of tissue that appears to extend continuously around

[7] See reference cited in footnote 4.
[8] See reference cited in footnote 5.
[9] The illustrations shown in plate 1, *A* and *B*, were made available through the cooperation of John D. Hunt, Division of Nutrition and Physiology, Bureau of Dairy Industry.

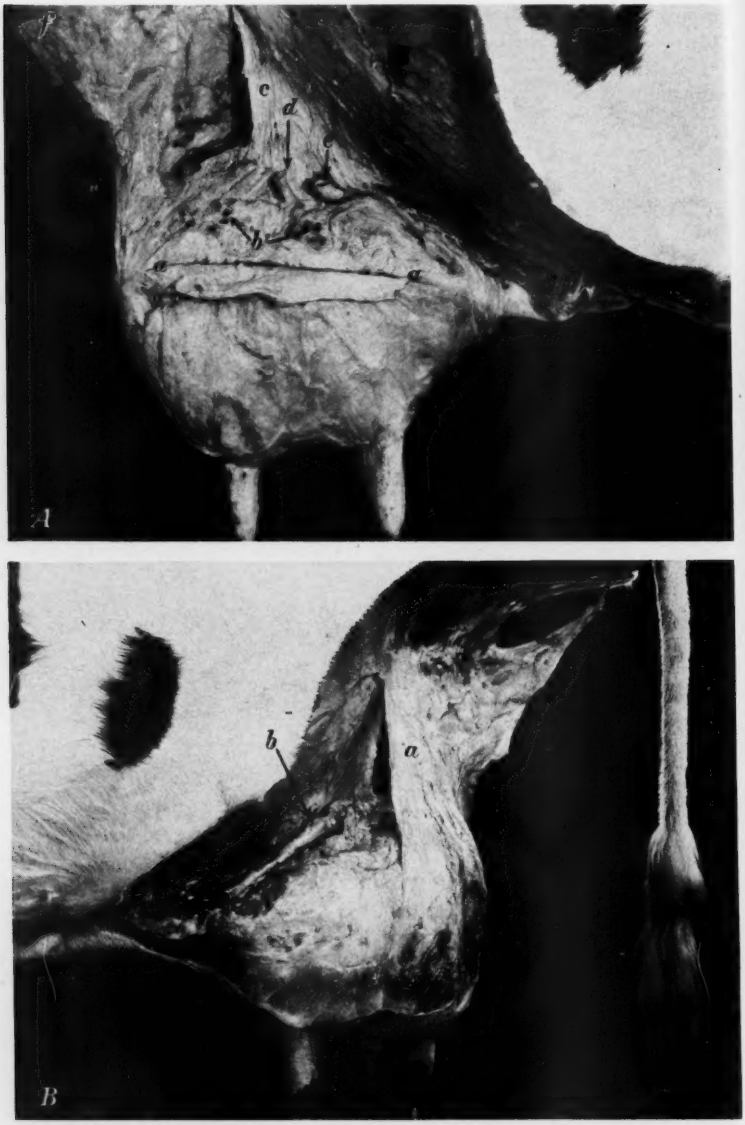

FIGURE 8.—*A*, Deeper dissection in which the deep lateral layer (tissue No. 5) is folded downward to a lower level revealing small areas of mammary-gland tissue (*b*), a portion of one of the median yellow elastic supporting sheets (*c*) (tissue No. 7), a large artery (*d*), and a large vein (*e*); the subpelvic tendon (*f*) (tissue No. 6) is still in position. *B*, Left side of udder with anterior part of deep lateral layer removed but with posterior part (*a*) still attached to udder and subpelvic tendon. A large vein is shown at *b*.

A, Lamellae (*a*–*a*) branching off from the deep lateral sheet (*b*) (tissue No. 5), and extending inward and downward to become continuous with the intraglandular tissues of the udder; *B*, similar lamellae (*a*–*a*) arising from one of the sheets (*b*) (tissue No. 7) which form the median septum; *C*, a band of tissue that appears to make the lateral (*a*) and median (*b*) layers (tissues No. 5 and No. 7) continuous around the ventral (lower) border of the gland (*c*).

the ventral (lower) border of the gland from the lateral to the median surface (pl. 1, *C*).

In figure 9, *A*, the udder is shown with all of the supporting tissues removed except a small area of areolar tissue and skin at the anterior (front) extremity of the mammary-gland tissue *b*, and the main portion of the deep medial, yellow elastic tissue *a* (tissue No. 7) which is attached to the abdominal wall above and continues below to form the septum between the halves of the udder. Apparently the areolar tissue and skin carried little weight. The white strip *c* between the abdominal wall and the dorsal surface of the udder is the result of light coming from the opposite side. It shows that all the supporting tissues in this area had been completely severed.

In figure 9, *B*, the udder is shown with all supporting structures removed except the main portion of the deep medial yellow elastic tissue *a* (tissue No. 7) that was pointed out in figure 9, *A*. The length (front to rear) of this layer of tissue, as shown, was 16.5 cm. (6.50 inches) where it attached to the abdominal wall, but was only 11.0 cm. (4.33 inches) at its shortest point. The great strength and the nearly perfect location of this median support are noteworthy. The weight of the udder, dissected as shown in figure 9, *B*, with the formalin it contained, was 47.0 pounds. Although the left half of the udder apparently was heavier than the right—a condition that caused the udder to list slightly to one side—its position when suspended from the short narrow sheet of median tissue as shown in figure 9, *B*, was otherwise not significantly changed from its position on the living cow. Measurements of the height of the tips of the rear and front teats were made from time to time as the dissection progressed. The heights measured at midafternoon on January 30, at the conclusion of the dissection shown in figure 6, *B*, were 37.5 cm. (14.76 inches) for the rear teats and 40.3 cm. (15.87 inches) for the front teats. Up to this time no settling or significant change in the position of the udder was apparent. After overnight suspension following the dissection shown in figure 6, *B*, the heights were 37.2 cm. (14.65 inches) and 39.7 cm. (15.63 inches), respectively, for rear and front teats. With the udder supported as shown in figure 9, *A*, the corresponding heights were 36.6 cm. (14.41 inches) and 39.5 cm. (15.55 inches) respectively, and after all of the supporting structures except the main portion of the medial yellow elastic septum had been severed (fig. 9, *B*) the heights were 36.3 cm. (14.29 inches) and 39.5 cm. (15.55 inches) for rear and front teats. These measurements indicate a total settling of only 1.2 cm. (0.47 inch) for the rear of the udder and only 0.8 cm. (0.31 inch) for the front since midafternoon of the previous day during which time all except one of the main supports of the udder had been removed. Obviously the median elastic tissue not only possessed great tensile strength, but it was so nearly perfectly located above the anteroposterior center of gravity that the udder was in almost a perfectly balanced suspension, when all other structures had been removed.

The results observed in this experiment appear to indicate that the medial septum is the principal structure by which the udder is sus-

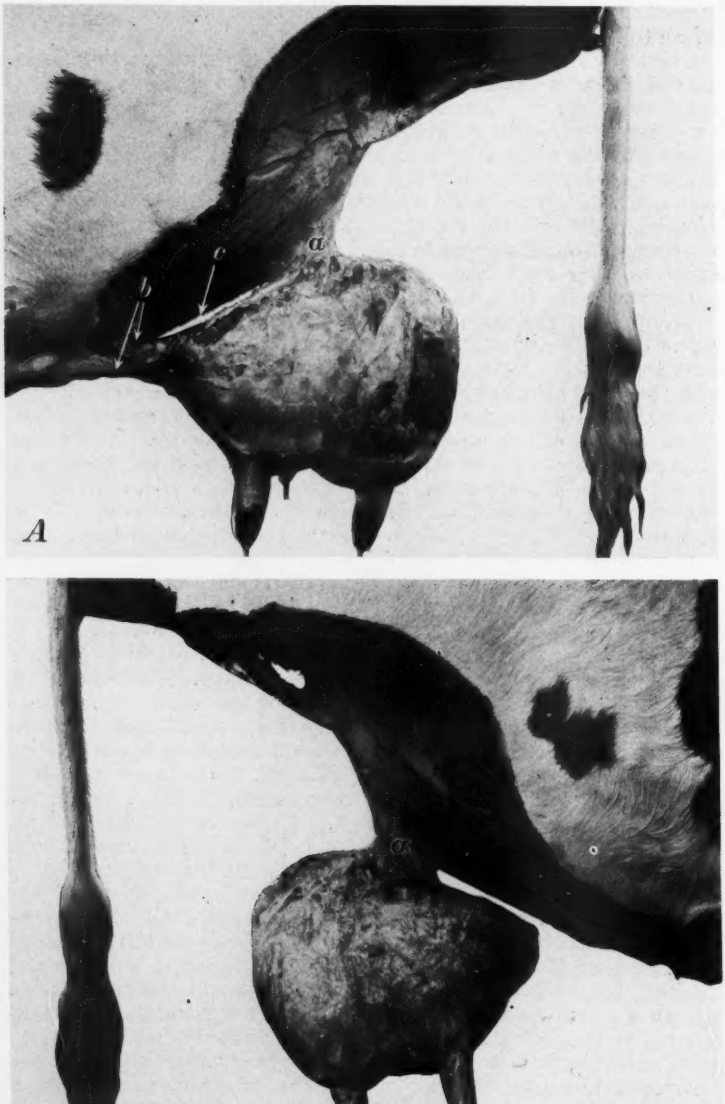

FIGURE 9.—*A*, Udder as seen from left, with all supporting structures removed except the main part of the medial yellow elastic tissue (*a*) (tissue No. 7) and a small area of skin and areolar tissue (*b*); a streak of light shows between the abdominal wall and the udder at *c*. *B*, View from the right with the medial yellow elastic tissue (*a*) remaining as the only support for the udder, note the almost perfectly balanced suspension of the udder.

pended. No other experiments were conducted to determine to what extent the other membranes could have supported the udder in position. It is of interest to note that the medial septum was attached to the abdominal wall but apparently not directly to any bony structure.

A rear and somewhat superior view of the udder at the same stage of dissection (fig. 10, *A*) indicates the narrowness of the main portion of the double sheet of medial tissue (tissue No. 7) shown in figure 9, *B*, which furnishes one of the chief supports of the udder and forms the septum that separates the two halves. The somewhat wider and heavier left half of the udder and the resulting swing of the entire organ to the right are clearly indicated.

At this point the right half of the udder was removed by means of a longitudinal incision that was carried as close as possible to the right of the median septum. Figure 10, *B*, shows the surface from which the right half of the udder was removed. It indicates the manner in which the supporting medial tissue (tissue No. 7) *a* spreads out in a fan-shaped manner to form a septum that reaches and attaches to nearly all parts of the medial flat surface of the gland. Apparently the fan-shaped distribution of this septum provides maximum support to the udder and accounts for its almost perfectly balanced suspension.

Figure 11, *A*, is a rear view that shows the shape and the appearance of the abdominal wall after the medial support of the udder was severed along the line *a*—*a*. In this view *b* shows a severed abdominal vein ("milk vein"), *c* is one of the ventral (lower) points of the symphysis pelvis to which the subpelvic tendinous support of the udder was attached, and *d* and *d* are the front legs of the cow.

Figure 11, *B*, is a lateral view of the abdominal wall at the same stage of dissection. It shows both the angle of inclination and the degree of curvature of the abdominal wall from the point at *b*, where the anterior (front) extremity of the udder had been attached, to the pubis at *c* (anterior part of pelvic floor). In this view a point of the symphysis pelvis (under median surface of pelvis) is shown at *d*, and the tendinous stump which formed the point of attachment for the median support of the udder is again indicated by *a*.

In addition to the photographs shown in figure 11 a number of measurements were taken to show the shape and position of the abdominal wall in the area where the udder had been attached. This study of the shape of the abdominal wall in relation to that of the dorsal (upper) surface of the udder was undertaken for the purpose of providing, if possible, some means of estimating the hidden boundaries of the udder and thereby of calculating udder volume in the living animal. The measurements were made with the embalmed cadaver still supported in the position shown in figure 2, *A*, with the udder and extraneous tissues dissected away as shown in figure 11. The measurements consisted of (1) horizontal distances from a perpendicular transverse plane located at the posterior extremity of the pinbone (ischium) to specified, previously located points; and (2) heights of the same points above the surface (of the stoneboat) to

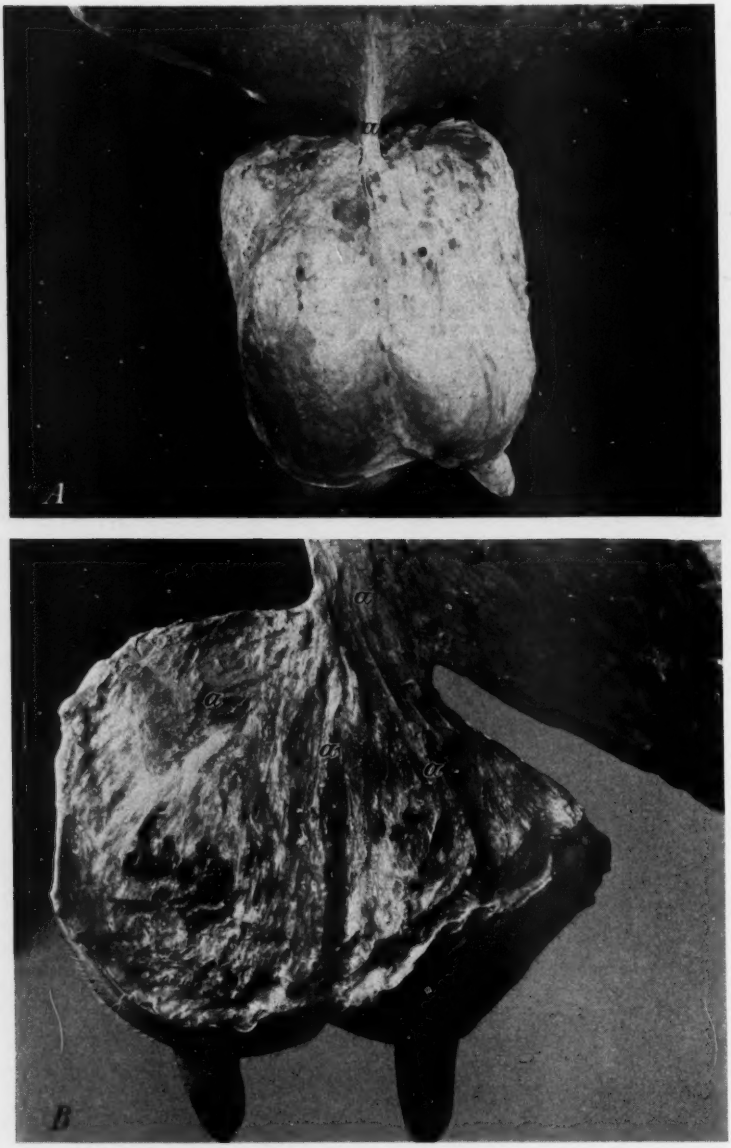

FIGURE 10.—*A*, Rear view of the udder from slightly above, showing the narrowness of the main part of the medial yellow elastic tissue (*a*) which is capable in itself of supporting the udder without material change of position; *B*, surface from which the right half of the udder has been separated to illustrate the fan-shaped attachment (*a*) of the septum to the medial surface of the udder.

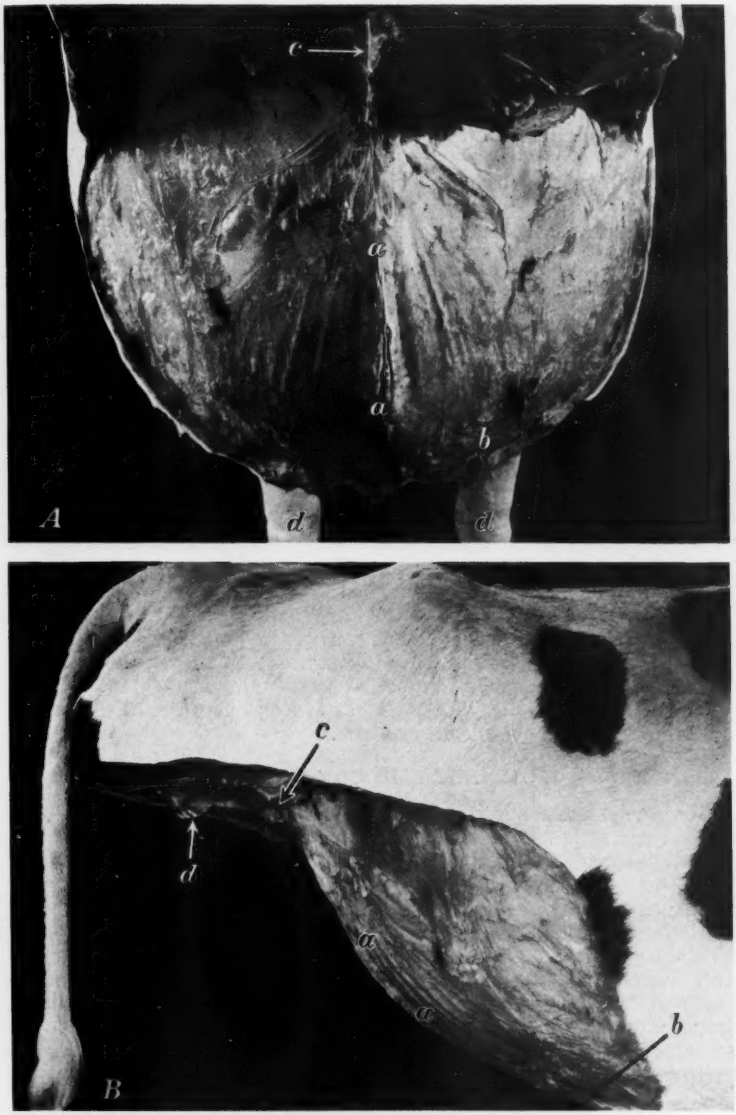

FIGURE 11.—*A*, Rear view of the abdominal wall after removal of udder: *a*—*a*, The line where medial septum was severed; *b*, a milk vein; *c*, one of the points at which the subpelvic tendon was attached to the pelvis; *d*, the front legs. *B*, Lateral view of the abdominal wall: *b*, The anterior point of attachment of the udder; *c*, the approximate location of the brim of the pelvis; *d*, the symphysis pelvis; and *a*–*a*, the tendinous stump of the medial yellow elastic supporting septum. Note that this medial septum was attached to the abdominal wall and not directly to any bony structure.

which the cow's feet were attached. The measurements obtained were as follows:

Measurement	*Centimeters*	*(Inches)*
(1) Anteroposterior length on the median line from a vertical transverse plane at the posterior extremity of the pinbone (ischium) to the intersection of the abdominal wall and a vertical transverse plane through—		
The navel	88. 50	(34. 84)
The anterior attachment of the udder	62. 00	(24. 41)
A front teat (right)	49. 50	(19. 49)
A rear teat (right)	35. 75	(14. 07)
(2) Height of the abdominal wall at the median line, above the surface to which the feet had been attached (stoneboat), in a vertical transverse plane through—		
The navel	56. 00	(22. 05)
The anterior attachment of the udder	62. 75	(24. 70)
A front teat (right)	73. 75	(29. 04)
A rear teat (right)	92. 00	(36. 22)

From these measurements the medial anteroposterior (front to rear) curvature of the abdominal wall was plotted. The plotted curve is shown in Figure 12, *A*, in which the line *o*—*x* represents the vertical transverse plane at the posterior (rear) extremity of the pinbone; *o*—*y*, the surface to which the cow's feet were attached (stoneboat); *a*, the navel; *b*, the anterior attachment of the udder; *c*, a vertical transverse plane through the front teats; and *d*, a vertical transverse plane through the rear teats. The distances from *o*—*x* and *o*—*y* to the points *a*, *b*, *c*, and *d* are shown in the graph.

By the use of special apparatus three contours were drawn (fig. 12, *B*) to show the transverse curvature of the abdominal wall at the vertical transverse planes passing through the navel (*a*), the anterior attachment of the udder (*b*), and a front teat (*c*). Plane *d* intersected the abdominal wall at a point too close to its junction with the pelvis to permit making a significant contour.

SUMMARY AND CONCLUSIONS

This study has made it possible to visualize and to illustrate the position and appearance of the principal structures by which the cow's udder is suspended. The fine areolar subcutaneous tissue by means of which the skin covering the udder is attached to the underlying tissues; the cordlike coarse areolar tissue which forms a loose connection between the upper surface of the front quarters of the udder and the abdominal wall; the superficial lateral sheets which arise from the subpelvic tendon, extend downward over the udder and attach to the thigh; the deep lateral sheets that have a similar origin but which virtually envelop the udder and attach directly to its outer surfaces by numerous plates that pass into the gland; the subpelvic tendon itself, from which the superficial and deep lateral layers arise; and the heavy yellow elastic sheets which arise from the abdominal wall, form a fan-shaped septum between the two halves of the udder, and serve as its chief median support and stabilizer, are all clearly shown in the illustrations that accompany the discussion.

Fleshy udders, udders that become edematous, and the udders of high-producing cows often become excessively large and heavy—particularly with advance in age—and overtax the structures by which they are suspended from the body. It is not difficult to imagine how, in these very large udders, any or all of the various sheets of supporting

tissues might become lax and stretch, and how the areolar tissue might pull apart in cases where any of the other tissues lose their normal tonus. It is likely also that a weakness in the suspensory

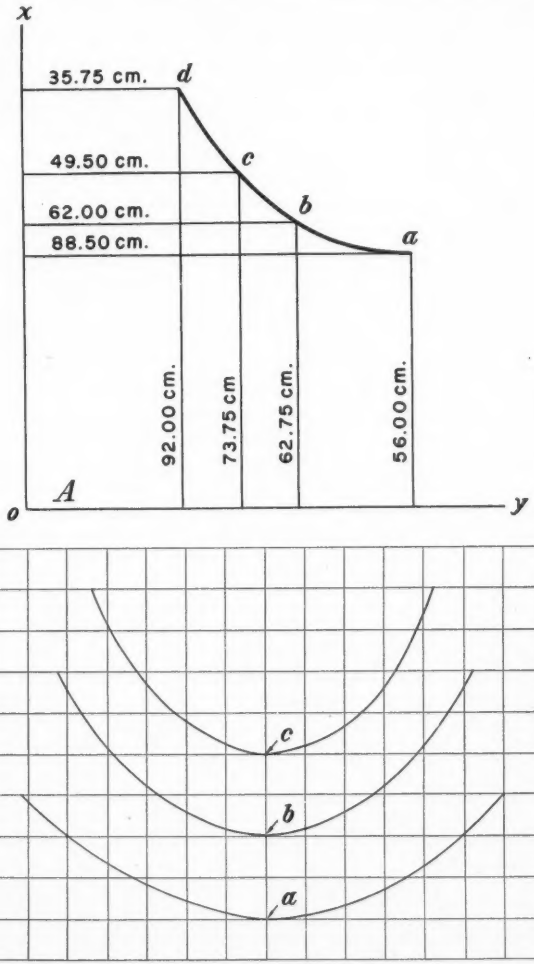

FIGURE 12.—*A*, Curve constructed from measurements to show the medial anteroposterior (front to rear) curvature of the abdominal wall from the navel (*a*) to the brim of the pelvis (*d*); measurements show distances from the floor and from the perpendicular at the rear of the pinbone (see text). *B*, The transverse curvature of the abdominal wall: *a*, At the navel; *b*, at the anterior (front) attachment of the udder; and *c*, directly above the front teats.

apparatus might occur as a result of inheritance or of a generally poor physical condition, and bring about a similar "breaking down" in udders that have not become excessively enlarged by fleshiness,

edema, or high production. The occurrence of poorly supported udders in young cows—sometimes early in first lactation—is an indication that such is the case.

The great tensile strength of the median elastic support of the udder (tissue No. 7), and its almost perfect location above its center of gravity are particularly noteworthy. In the case studied the main portion of this median support, which was less than 4½ inches long (front to rear) at one point, and had only a 6½-inch attachment to the abdominal wall, was capable in itself of holding the udder in an almost perfectly balanced suspension. Undoubtedly its fan-shaped attachment to the medial faces of each half of the udder accounted largely for the balance of the udder.

It probably is this median supporting tissue that becomes excessively relaxed in cases where the udder sags along the median line and the teats point outward laterally. It is likely also that a lack of tonus in the lateral sheets of supporting tissue (tissues No. 4 and No. 5), together with a separation of the fibers of the areolar tissues (tissues No. 2 and No. 3), may cause the udder to break down on the sides (front, rear, or both) with the result that the active support becomes very narrow and appears to be almost entirely median.

The dorsal (upper) surface of the udder does not, as has sometimes been supposed, follow a continuous curve from its anterior to its posterior attachments. It appears to follow quite closely the curve of the abdominal wall toward the rear to a point approximately under the brim (front) of the pelvis, but subsequently it carries out in a generally horizontal plane or may incline downward. In the case of the cow studied the dorsal surface of the udder posterior to the brim of the pelvis inclined rather definitely downward toward the rear. In this connection the poor rear attachment of the udder in this animal both as a calf and as a cow is again pointed out.

It is noteworthy that in the cow dissected the junction of the abdominal wall and the pubis—almost directly below the acetabulum (hip joint)—was located superiorly (above) and essentially in a vertical transverse plane passing through the rear teats. Presumably the relative location of the teats with reference to these points will vary with individual cows, depending largely on the shape and position of the udder.

The study of this cow emphasized the fact that the area of contact between the dorsal surface of the udder and the abdominal wall was very much smaller than the area of a horizontal transverse section through the udder.

Owing to the fact that the dorsal (upper) surface of the udder does not attach directly to the abdominal wall, or coincide with it in shape, posterior (to the rear) to a plane approximately at the midpoint of the udder, it is doubtful whether the curvature of the abdominal wall can be used as a reliable guide in estimating the size or volume of the udder in the living cow. The difficulty in making an accurate estimate of udder volume is complicated also by the fact that the dorsal (upper) contour of the udder is dependent to a very great extent on the quality and nature of its suspensory apparatus, which appears to vary greatly in individual animals. Moreover, such an estimate is likely to be inaccurate on account of the possibility that laxity in the abdominal muscles may alter the shape and position of the udder.

This study, by illustrating the form and nature of the apparatus by

which the udder is attached to the cow's body, should make possible a better understanding of the conditions that may exist and of the anatomical changes that may have taken place, as a result of an udder becoming "broken down" and pendulous. If, as it appears, an inherited weakness in the suspensory tissues, and excessive weight of the udder and its contents are two of the chief factors contributing to the breaking down of the udder, the selection of breeding stock that are known to have an inheritance for well attached udders, and more frequent milking of heavy producing cows, should be effective as preventive and remedial measures.

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A MICRURGICAL STUDY OF CROWN GALL INFECTION IN TOMATO[1]

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INTRODUCTION

This paper is concerned with micrurgical studies on crown gall infection in tomato (*Lycopersicon esculentum* Mill.), with emphasis on the relation of inoculation technique to infection. Despite a voluminous literature on crown gall (*11*),[2] little is known of the quantitative aspect of the infection mechanism, particularly the limits in wound size and in bacterial numbers that govern infection.

LITERATURE REVIEW

Levine (*9*) studied the effect of inoculating tobacco plants with various quantities of different dilutions of the crown gall organism (*Phytomonas tumefaciens* (Sm. and Town.) Bergey et al.). He used suspensions ranging from ordinary agar culture washings to dilutions of 1:100, but he found no marked difference in the size of the galls resulting from any of the preparations. Manifestly, however, even in the 1:100 dilution, the number of bacterial cells must have been in the thousands. The results of this work emphasize the limitations of the dilution method and the indispensability of micrurgical or micromanipulative technique in studies involving small numbers of micro-organisms.

In phytopathology, while the most extensive application of micrurgy has been in connection with the isolation of single cells to obtain pure cultures (*4*, *6*), other applications have involved pathological studies of bacterial, virus, and fungus diseases (*1*, *5*, *7*, *8*, *10*, *12*, *13*, *14*). Hildebrand (*5*) was the first to demonstrate that a single bacterium is capable of inducing disease in plants.

In animal pathology, although there are reports that as few as one individual tuberculosis germ can produce infection, Webb, Williams, and Barber (*17*) induced tuberculosis in a guinea pig by subcutaneous inoculation with a minimum of 20 tubercle bacilli. They refer to work by Wyssokowicz in which he reported that 8 tubercle bacilli were able to set up an infection in the peritoneal cavity of the guinea pig and that 24 to 30 bacilli were required in the rabbit. They also report that on 4 different occasions 1 thread (3 to 6 bacilli), isolated directly from the blood of a mouse dying from anthrax, caused death when inoculated into healthy mice.

[1] Received for publication November 1, 1941. The work reported here was begun in 1939 at the Rockefeller Institute for Medical Research, Princeton, N. J., during tenure by the writer of a Guggenheim fellowship. The writer was on sabbatical leave from Cornell University in 1939–40, and the work was later completed at Cornell. All the diagrams were prepared by Mrs. D. W. Thomas.

[2] Italic numbers in parentheses refer to Literature Cited, p. 58.

Thöni and Thaysen (*15*), employing between 10 and 343 tubercle bacilli per inoculation dose, were able to get infection in guinea pigs in but 1 instance, with 71 cells. Although the results of these investigators seem to contradict those of earlier work, it should be mentioned that they used Burri's (*3*) india-ink method, which because of optical difficulties has rarely been used for isolating bacteria, whereas the method of Barber (*2*), employed with some modifications by Webb, Williams, and Barber (*17*), remains even today perhaps the most reliable and widely used technique for isolating single micro-organisms (*6*).

In 1926 Wâmoscher (*16*) conclusively demonstrated for the first time the infectivity of single pneumococcus cells in mice, when 5 out of 21, or 23.8 percent, of the mice that received single cells died from the disease. He obtained a maximum infectivity of 83.3 percent death, with dosages consisting of 11 to 20 bacterial cells per mouse. In making isolations of single cells from blood he used a Peterfi micromanipulator and dark-field condenser. For inoculation the pipette was introduced into a wound in the skin and the tip was broken off underneath the skin surface with a tweezer.

The present paper, an abstract of which has already been published (*8*), reports the results of micrurgical studies with the crown gall organism and attemps to evaluate the significance of wound size and bacterial population in the infection mechanism.

MATERIALS AND METHODS

Bonny Best tomato plants selected for size and uniformity (about 6 inches tall) were used in all experiments.

Several crown gall cultures were used but the chief reliance was placed on the peach strain of N. A. Brown, obtained from Dr. A. C. Braun of the Rockefeller Institute. Highly motile young cultures grown in nutrient media for 9 to 15 hours were ordinarily employed.

The micrurgical apparatus consisted of a double Chambers micromanipulator and accessories (*6*) in modified arrangement for isolation of the bacteria under one microscope and their immediate transfer to the infection court of a plant under a second microscope. Inoculation was accomplished either by a simple shift of the micromanipulator from one microscope to the other or by removal of the pipette containing the inoculum from the holder and manipulating it in the hand during inoculation.

Single-cell isolation was accomplished with the method described by the writer (*6*). By this method one or any desired number of young motile cells could be isolated in preparation for transfer to infection courts.

The smallest wounds tested as infection courts were made into individual living cells in the intact tomato plant with a pipette having a tip diameter of about 3 μ (figure 1). The basal cells in the trichomes or large hairs and the largest epidermal cells adjoining the basal cells of the trichomes on the petiole or stem surface of the tomato were chiefly employed. India ink and olive oil also were injected into living plant cells to check the value of the technique as a means for introducing materials.

The next larger size of wound tested as an infection court was produced by gently stroking the tomato stems once with the side of a polished dissecting needle previously dipped into a bacterial sus-

pension (figure 3, *A*). By this means the trichomes as well as the smaller glandular hairs in the rubbed strip were crushed and small wounds produced both on the hairs themselves and where they joined the epidermis underneath. This operation was performed under a binocular dissecting microscope. The injuries produced appeared to be limited to one or a small number of cells at the base of the hairs. Stems stroked but not inoculated later showed superficial light-colored scars where the hairs had been removed, indicating the very limited character of the injuries.

This method of inoculation was chosen only after nine different ways of producing tiny wounds in individual plant cells or in very small numbers of plant cells on the stem surface had been tried. These

FIGURE 1.—A microinjection pipette inserted into the basal trichome cell on a tomato stem. ×50.

methods of wounding, tested on material under the microscope, were as follows: (1) pinching off the tips of trichome hairs approximately 2 cells away from their basal stem attachment with tweezers that had previously been dipped in a bacterial suspension; (2) pinching off trichomes back to 1 cell away from the stem attachment; (3) removal of trichome hairs with a tiny razor so as not to injure the neighboring basal cells; (4) pulling off trichome hairs with square-tipped tweezers, which often resulted in injury to 1 or more of the neighboring cells; (5) gently stroking the tomato stem with the side of a polished dissecting needle; (6) more vigorous stroking of the needle on the stem surface so as to cause collapse of hairs and injury to epidermal cells underneath; (7) making very shallow wounds by puncturing the epidermis with a small dissecting needle to the depth of 2 to 4 cells; (8) using a similar needle for puncturing the epidermis to a depth of 5 to 8 cells; and (9) using a similar needle for puncturing to a depth of 10 cells or more.

In preliminary tests of methods 1 to 6 the wounding instrument was always dipped in a bacterial suspension so that the amount of inoculum applied was not controlled, although the number of bacteria that got into the microscopic wounds was undoubtedly very small.

Wounding large hairs by methods 1, 2, and 3, always gave negative results, which supports the findings of earlier tests in which individual cells of trichome hairs were injected with bacterial suspensions. Occasional tiny galls resulted in from less than 1 to 10 percent of the trials in wound types 4, 5, and 6, with greatest incidence in 6, where most injury was produced.

Wound types 7, 8, and 9 resulted in gall formation in a relatively higher percentage of cases, apparently because these wounds were larger than the minimal size necessary for infection as represented in wound types 4, 5, and 6.

Deep wounds, to depths of approximately one-fourth, one-half, and completely through the stem, produced under the microscope, were tested as infection courts for different amounts of inoculum. The side of the stems receiving such wounds, as well as those of types 7, 8, and 9, were stroked previously with a smooth needle so as to remove the pubescence, an obstruction which interfered with working on shallow needle-puncture wounds. Following the withdrawal of the needle from both the shallow and deep needle-puncture wounds, sterile juice extract was added from a pipette to provide a protruding meniscus to the wound cavity for facilitating the transfer of inoculum. Immediately after inoculation the plants were placed in a moist chamber for approximately 1 hour to retard the drying of the wound and to allow for penetration by the bacteria.

Sterile juice extract for the purpose indicated and for testing as a growth medium was prepared from the foliage of young, rapidly growing plants. The material was ground in a food chopper, extracted through cloth, centrifuged, filtered through a bacteria-proof filter, and then used immediately or stored in a refrigerator until used.

EXPERIMENTAL RESULTS

GROWTH MEDIA

Juice Extract From Tomato

Sterile juice extract prepared from the stems and leaves of young tomato plants was found to be an excellent medium for culturing single cells of the crown gall organism. Approximately 90 percent of the single cells grew in microculture when transferred to small droplets of the extract of three different preparations of sterile juice. The isolation technique earlier described by the writer (*6*) was used. Three such experiments were conducted which involved the transfer of 9, 11, and 10 active young (10 to 15 hours old) single bacterial cells to as many microdroplets, of which 8, 10, and 10 grew.

The fact that single cells of the crown gall organism grew and multiplied readily in the juice extract from tomato plants indicated that individual bacterial cells should be able to grow also in the plant sap contained in wound cavities of the tomato and possibly even within individual plant cells if such an environment should prove to be congenial and a suitable inoculation technique could be developed.

Wound Sap

Proof that single cells of the crown gall organism had multiplied in the sap in wound cavities were demonstrated by isolation experiments. Five days after deep wounds had been inoculated with single bacterial cells, and before visible evidence of gall formation, wound tissue was dissected from 20 plants, crushed in distilled water in Petri dishes, and poured with nutrient dextrose agar. Seven days later bacterial colonies like those produced by the crown gall organism were noted on 6 plates. In 1 plate there were about 50 colonies, but in the other 5 the colonies were too numerous to count and were estimated to be in the thousands. Representative colonies were inoculated into tomato plants with positive results, leaving no doubt as to the identity of the organism.

As an outgrowth of these preliminary experiments the author set out to discover, if possible, (1) the smallest size of wound that could be used for infecting tomato plants, (2) the smallest number of crown gall bacteria that would produce infection, and (3) the relative influence of number of bacteria in the inoculum and size of wound on the ultimate size of the galls.

SMALLEST SIZE OF WOUND REQUIRED FOR INFECTION

Microinjection into Individual Plant Cells

One hundred and fifty individual plant cells (120 trichome hair cells and 30 epidermal cells) were inoculated by injection with a bacterial suspension (table 1). The micropipette used for inoculation was introduced into the plant cells under the control of a micromanipulator (fig. 1). When the cell wall was perforated with the pipette tip a hemispherical droplet of protoplasm gushed out and then quickly returned into the cell. At the precise moment that the latter process

Table 1.—*Summary of infection experiments involving various types of wounds and amounts of inoculum*

Kind of wound	Tool used	Bacteria used as inoculum	Experiments	Plant cells or stems inoculated	Crown galls induced			Range in radial extension
					Number	Percentage	Range between experiments	
		Number	*Number*	*Number*			*Percent*	*Millimeters*
Microwound (3 μ ±)	Micropipette.	Many	10	150 cells	0	[1] 0		
Microwounds (injury to 1 or more epidermal cells).	Polished needle.	Many	10	500 stems	Many	[2] 5.0		1-2
Shallow wounds (2-4 cells, 5-8 cells, and 10-12 cells deep) grouped [3]	do	1	10	102 stems	10	10.0	0-25	1-7
		2	9	80 stems	8	10.0		
		5	9	72 stems	10	14.0	6-30	
		10	9	90 stems	18	20.0		
		50-100	5	43 stems	9	21.0	10-36	
Deep wounds (one-fourth through, one-half through, and entirely through stem) grouped [3]	do	1	4	40 stems	13	32.0	10-60	10-60
		2	4	40 stems	12	30.0		
		5	4	40 stems	18	45.0	20-90	
		10	4	40 stems	26	65.0		
		50-100	4	40 stems	39	97.5	90-100	

[1] Viable bacteria isolated from only 1 out of 20 tested for survival in inoculated trichome hairs.
[2] Estimated percentage of tiny wounds infected.
[3] The different depths of wounds were grouped together because in failing to control the depth of penetration of the inoculum into the wound by the technique used wound depth lacked significance.

took place increased pressure was put on the pipette contents so as to expel a quantity of the bacterial suspension into the plant cell. Almost immediately after the withdrawal of the pipette what appeared to be a protoplasmic plug filled the wound opening and effectively protected the cell from loss through evaporation. A droplet of india ink was placed on the stem surface adjacent to the inoculated trichome to facilitate its location afterward. Such inoculated cells appeared not to be injured and practically always remained alive, as judged by the fact that protoplasmic streaming continued after the operation. In a few instances particles resembling bacteria could be seen moving in the protoplasmic stream within trichome cells in much the same fashion as do india-ink particles when injected into similar cells.

Not a single instance of gall formation was found in these tests when final observations were made (fig. 2), and 3 weeks after inocula-

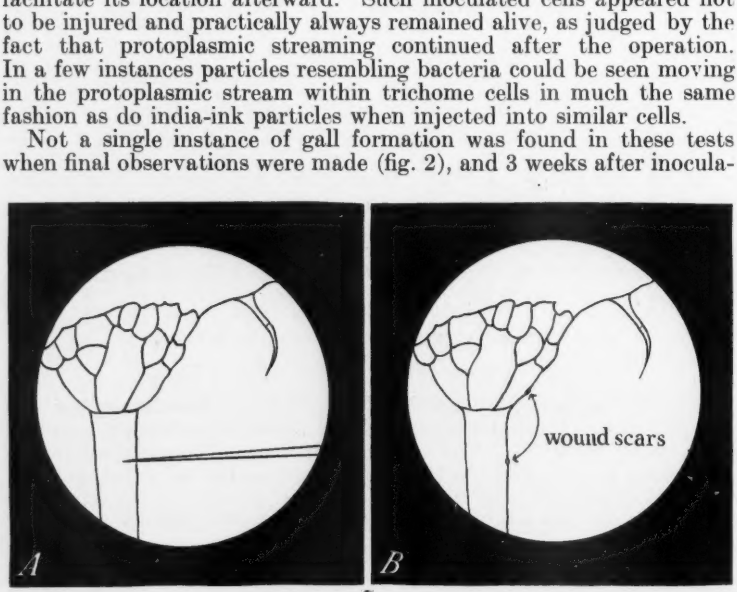

FIGURE 2.—Microinjection of individual trichome cells of tomato with crown gall bacteria: *A*, Many bacterial cells being injected into an individual cell (trichome or epidermal); *B*, at the end of 3 weeks no galls have resulted from the injection and no viable bacteria have been recovered.

tion, many of the trichome cells were still functioning normally. To account for these results several explanations are possible: (1) Wounds approximately 3μ in size may be too small to permit infection if it be assumed that a certain minimum quantity of wound hormone is essential for gall formation; (2) the intracellular environment may not be a favorable medium for these bacteria; (3) the injected bacteria that survived may have been too few or the period of survival too short for stimulating gall formation; or multiplication of bacterial cells, which apparently did not occur, may be essential for gall formation.

ISOLATIONS FROM INOCULATED TRICHOMES

Three weeks after inoculation, virulent crown gall bacteria were recovered from injected trichomes in but 1 case of 20 cultured. The isolations were attempted from 7, 6, and 7 living trichomes from 3 separate inoculation experiments and the crown gall organism was cultured from but 1 trichome in the first series. The hairs were removed by means of a tiny razor soldered to a needle and crushed in a droplet of broth on the inside of a test tube before washing into

a small quantity of sterile broth at the bottom. The interval of 3 weeks was thought to be sufficient for eliminating chance organisms left outside the wound opening in the inoculation operation. However, the single positive case may be the exception to the rule. Since bacteria were isolated in only 1 instance in 20 attempts it would seem that the intracellular environment was not a favorable medium for survival.

Tiny Galls Produced at Tiny Stem Wounds

The smallest wounds in which infection occurred were produced by gently stroking the stems and petioles of tomato plants with a smooth polished needle previously moistened in a bacterial suspension of the crown-gall organism (fig. 3).

Within 5 days after inoculation tiny galls began to appear in close proximity to collapsed hairs and apparently arose from wounds involving one to several epidermal cells. The galls ranged from microscopic size to about 1 mm. in radial extension. Maximum size was reached within about 3 weeks, and forcing the plants by fertilization and planting in deep soil failed to materially increase the size of the galls even after 3 months.

It was estimated that less than 5 percent of the plant cells injured in this experiment became infected. The extent of the injuries depended on the pressure used in stroking and the number of times the needle was passed over a given point. The rubbing operation simultaneously applied a film of bacterial suspension and caused the extrusion of wound sap because of the pressure applied. As soon as

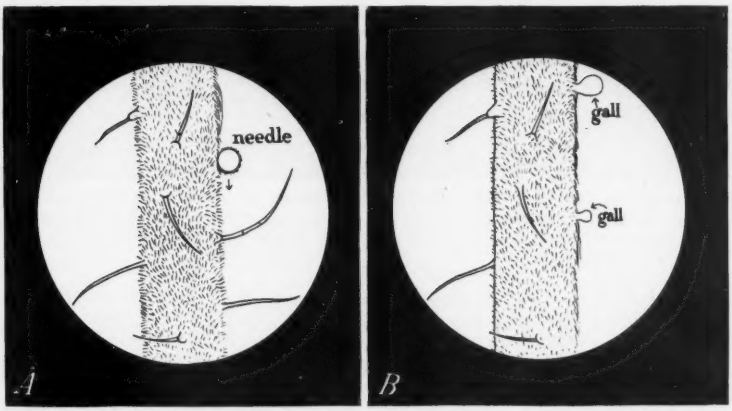

Figure 3.—Rubbing technique of inoculation: *A*, A needle dipped in bacterial suspension was stroked gently over the stem surface, resulting in minute wounds involving one to several plant cells; *B*, occasional tiny galls formed in proximity to the collapsed hair cells, such galls reaching a radial extension of about 1 mm.

the pressure was removed some of the sap and bacterial mixture returned into the wound.

From the results obtained it appeared that the small size of the galls may have been due to the minuteness of the food supply in the wounds and the relatively few bacterial cells that could gain entry and survive therein.

Bacterial Population in Tiny Galls

Estimates of the number of bacterial cells were obtained by the standard dilution agar plate technique. Individual galls of several ages and sizes were used with or without a washing treatment to remove the bulk of chance organisms present on the outside. Excess water was blotted off the galls by means of sterilized paper toweling and entire galls were removed, cutting tangentially to the stem surface, with a razor. Each gall was ground fine in a sterile mortar in a small quantity of water before dilution plates were poured. It was soon found that dilutions were ordinarily unnecessary because of the small yield of bacteria. After an incubation period of about 4

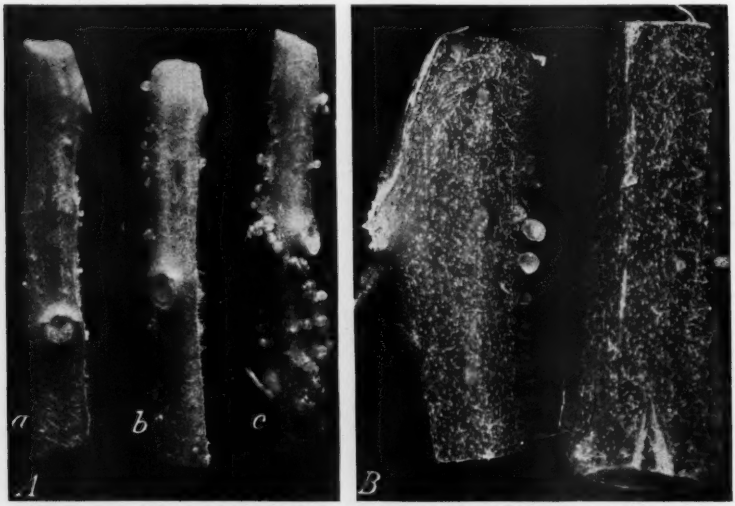

Figure 4.—*A*, Tiny galls induced by rubbing tomato stems with a polished needle gently (*a*) and progressively harder (*b*) and (*c*). The increase in size of wounds was correlated with an increase in the size of the galls induced. ×0.9. *B*, Enlarged view of two stem pieces with similar tiny galls. ×3.

days colonies resembling the crown gall organism were counted, and representative samples were used for pathogenicity tests to confirm the diagnosis.

On the whole, the number of bacteria isolated from tiny galls was very small, averaging less than 100. In 1 series of experiments the 54 galls cultured gave a total of 1,691 colonies, or an average of 31 colonies per gall, while the yield per gall ranged from 0 to 550 colonies. Only 4 galls yielded over 100 colonies. In another study 90 tiny galls yielded a total of 10,582 colonies or an average of 118 colonies per gall. Of these 1 gall yielded 5,520 or over half of the total; a second gall gave 1,223 colonies; 7 galls gave between 100 and 1,000 colonies; 34 galls less than 10 colonies; and 29 galls gave none. Failure to obtain any bacteria in about one-third of the galls suggests their elimination either through the technique employed or by death due to an unfavorable environment. It is possible that some of the galls negative for bacteria in the isolation tests are representative of what

is obtained when crown gall bacteria get inside living tomato cells or, as in the case of sunflower (*18*), when bacteria-free secondary galls occur at a distance from the point of inoculation. While the inability of crown gall bacteria to survive within injected cells, already mentioned, supports this conclusion, more work is needed on this point.

Location of Bacteria in the Galls

The approximate location of the crown gall bacteria in the tiny galls was studied by pouring plates of galls divided into two or three parts under the microscope by means of a microtool fashioned from a safety razor blade. Three experiments were conducted.

In the first experiment isolations were attempted from the tip one-third part of 7 tiny galls, and the results were positive in 2 cases. At the same time isolations made from 11 complete galls gave positive results in all cases.

The second experiment involved isolations from 10 tiny galls divided into 2 approximately equal parts. All galls yielded bacteria—the tip half in 9 cases and the basal half in 4 cases.

In the third experiment eight galls were divided into three approximately equal parts. Of the eight galls seven yielded pathogenic bacteria. Bacteria were obtained from the tip, middle, and basal regions respectively of three, five, and four of these tiny galls.

The results of the foregoing tests show that bacteria may be found in various parts of the tiny galls and are not limited to one location.

Smallest Number of Bacteria Required to Induce Gall Formation

Shallow Needle-Puncture Wounds

When one or more bacterial cells were used as inoculum and shallow stem wounds (about 0.1 mm. in diameter) of three depths (2 to 4 cells deep, 5 to 8 cells deep, and 10 to 12 cells deep, approximately) were employed as infection courts, infection occurred in a maximum of 21 percent of the trials (table 1).

Single bacteria induced gall formation in about 10 percent of the plants inoculated, as compared with approximately 15 percent for 2 to 10 bacterial cells and 21 percent for 50 to 100 bacterial cells (fig. 5).

The galls induced by single cell inoculation began to appear in about 1 week after inoculation and reached maximum size in 3 weeks. They were usually small, ranging from less than 1 to about 4 mm. in radial extension at the end of 3 months (fig. 6*A*). There was a tendency for the galls to become larger as the depth of the wound increased.

The galls induced by inoculation with 2 to 10 bacterial cells finally reached between 1 and 5 mm. in radial extension. Again the larger galls were associated with the deeper wounds.

When between 50 and 100 bacterial cells were introduced into shallow wounds the majority of the resulting galls reached between 1 and 7 mm. in radial extension and were larger for the greater wound depths.

From these results it is apparent that infection was somewhat higher and the galls were larger when more than one bacterial cell was used as inoculum and deeper wounds were inoculated. This slightly higher percentage of infection where more than one bacterial cell was used, while significant, is believed to be the result of chance. The probability of a single bacterium, as contrasted with two or more cells, reaching

the proper location in the wound for setting up an infection was undoubtedly less; therefore the percentage of infections would be expected to be smaller for the single cell. Moreover, one or more cells of an inoculum consisting of several cells would have a better chance of reaching a greater depth, as well as a better position for multiplication, than would a single cell. Consequently a correlation between size of gall and depth of wound was expected. Basic to the above interpretation is the well-known tendency of the crown gall

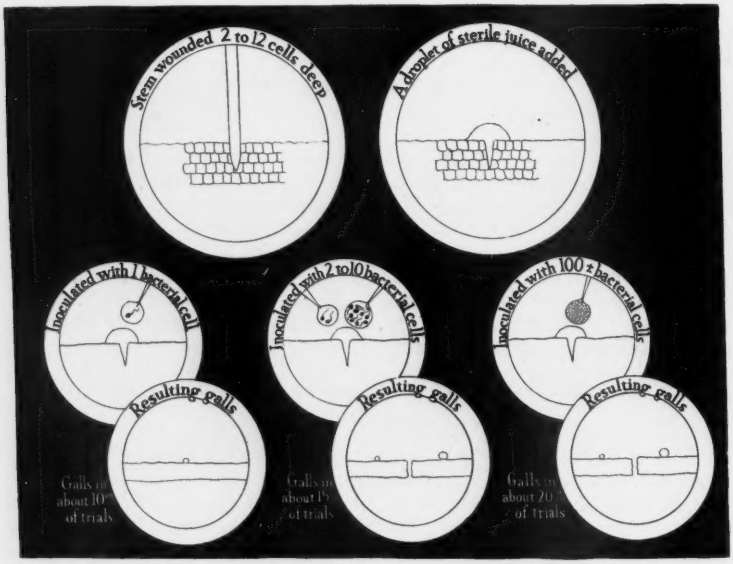
Stem wounded 2 to 12 cells deep
A droplet of sterile juice added
Inoculated with 1 bacterial cell
Inoculated with 2 to 10 bacterial cells
Inoculated with 100 ± bacterial cells
Resulting galls
Resulting galls
Resulting galls
Galls in about 10% of trials
Galls in about 15% of trials
Galls in about 20% of trials

FIGURE 5.—Inoculation of shallow needle-puncture wounds with one or more crown gall bacteria, showing production of small galls.

organism to localize in the tissue and to stimulate only a limited number of the surrounding cells to activity.

DEEP NEEDLE-PUNCTURE WOUNDS

Deep wounds made by using the same size needle as before proved much more efficient as infection courts than shallow wounds (table 1). Three types of deep wounds (needle punctures one-fourth, one-half, and completely through the stems) were tested, one or more bacterial cells being used for inoculum. Single bacteria produced infection in from 10 to 60 percent of the trials (fig. 7); 2 to 10 bacteria produced galls in from 20 to 90 percent of the trials, and 50 to 100 bacteria produced galls in practically all the trials. In every case the galls were larger in radial extension than the thickness of the stems. There was a definite correlation between depth of wound and size of gall since the largest galls were always associated with needle punctures all the way through the stems (fig. 6, *B*). However, no apparent correlation was found between amount of inoculum and

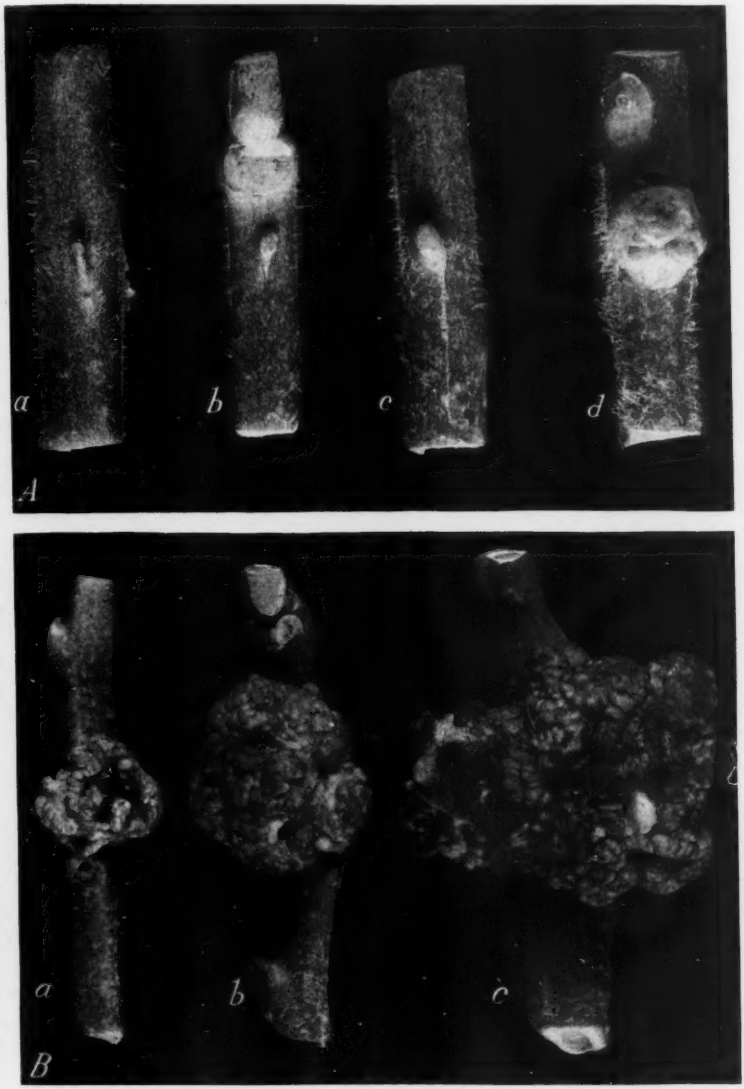

FIGURE 6.—Crown galls induced by inoculation with single bacteria: *A*, Maximum size attained in 3 months by crown galls induced by single-cell inoculation into wounds 2 to 4 cells deep (*b*); 5 to 8 cells deep (*c*); and 10 to 12 cells deep (*d*), as compared with a tiny gall (*a*) induced by inoculation of a very small epidermal wound resulting from the removal of a single trichome hair. × 1.4. *B*, Maximum size attained at the end of 3 months by galls induced by the introduction of single bacteria into wounds one-fourth (*a*), one-half (*b*), and completely through (*c*) the tomato stem. × 0.9.

size of gall. The reason for the single cell being less infective than larger numbers of cells on a percentage basis was undoubtedly due to the fact that there was less chance of one cell finding a favorable place for multiplication in the wound cavity. However, if a bacterium once found congenial surroundings, as in a deep wound, it proved actually equal in potentialities to larger numbers of cells so far as gall size, the end result, was concerned.

It has already been shown that one bacterium can quickly multiply into large numbers in sap extract and that the sap in the wound cavity functions in the same way as the food supply in microculture or in a test tube. The principal limiting factor governing gall size in these experiments was food supply for the increase of the bacterial population and not the initial amount of inoculum. Therefore, it was

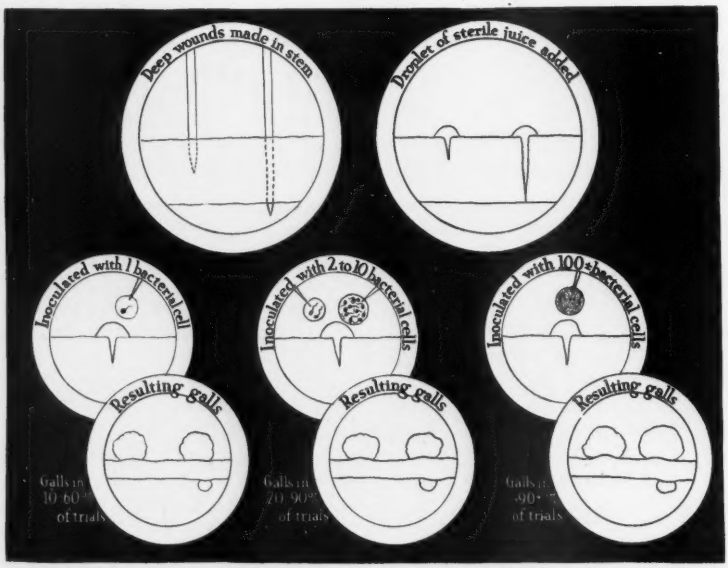
Deep wounds made in stem
Droplet of sterile juice added
Inoculated with 1 bacterial cell
Inoculated with 2 to 10 bacterial cells
Inoculated with 100± bacterial cells
Resulting galls
Resulting galls
Resulting galls

FIGURE 7.—Inoculation of deep needle-puncture wounds with one or more crown gall bacteria showing production of large galls.

concluded that the kind of wound was a more important factor in producing infection than the initial number of bacteria.

BACTERIAL POPULATION AND SIZE OF GALL

Isolations made from galls of various sizes taken at random from the several experiments in which shallow and deep needle-puncture wounds were employed indicated that bacterial population is roughly proportional to gall size. Small galls ranging from 1 to 3 mm. in radial extension yielded an average of between 50 and 100 colonies per gall in isolation trials. Always the entire gall was crushed in water in a Petri dish and allowed to stand for an hour before the agar was added. Galls from 5 to 7 mm. yielded between 100 and 50,000 colonies.

Galls 7 mm. and upwards ordinarily gave counts averaging 100,000 or more. One series of 7 galls measuring from 10 to 25 mm. in diameter yielded in round numbers 1,106,500; 400,000; 1,143,000; 1,070,000; 510,000; 11,000,000, and 20,000,000 bacteria based on the plate counts.

DISCUSSION

The present investigation, which involved the use of micrurgical technique, demonstrated the fact that a single cell of the crown gall organism when introduced into a wound is capable of producing infection in tomato. Apparently such potentiality of individual bacteria had been previously demonstrated in but one instance in animals (*16*) and one in plants (*5*).

The greatest difficulty encountered in this study was not that of developing precision technique for isolating and transferring known numbers of bacteria to the wounds, but rather that of introducing the isolated bacterial cells into the proper position in the needle-puncture wounds for infection to take place. Filling the wounds with sterile juice extract and then planting the inoculum on the protruding meniscus was found to be inadequate for the proper distribution of inocula in the shallow wounds, but proved much more efficient for the deep wounds. The interpretation given to this seeming discrepancy was that of the role of chance in distributing the bacteria in the wounds.

The complete failure of microscopic pipette wounds to become infection courts probably cannot be charged to lack of perfection of the microinjection technique. This conclusion is supported by the evidence from isolation experiments in which it was found that virulent cultures failed to survive the intracellular environment. Thus far no one has given convincing evidence that living crown gall bacteria are ever present in living plant cells. One of the most recent studies was that of Banfield (*1*) who reported negative evidence on this point. The final answer to this question will require further work.

SUMMARY

Juice extract from tomato plants was found to be an excellent medium for culturing the crown gall organism. Single bacteria grew readily in the juice extract in microculture.

The sap in the wound cavity liberated from the cells that were injured in the wounding operation also supported growth of the crown gall organism. Isolations made from wounds about 5 days after inoculation and before symptoms appeared, showed that the original single cells had multiplied into thousands of individuals in the wound sap.

The crown gall organism, when injected into the living cells of tomato stems, failed to induce gall formation and ordinarily failed to survive inside the cells, indicating that the living cell interior is an unfavorable medium for these bacteria.

Single bacterial cells induced gall formation when introduced into needle-puncture wounds of various sizes. The lower percentage of infections resulting from single-cell inoculation as contrasted with that from inocula consisting of larger numbers, was attributed to the role played by chance in distribution which favored the larger num-

bers reaching the proper position for multiplication and cell stimulation in the wounds.

Tiny wounds in the tomato stem, involving one or more epidermal cells, approximated the minimum size for infection by the crown gall organism. Only a small percentage of such wounds became infected when the inoculum was applied by gently rubbing the stem surface with a polished needle moistened with a bacterial suspension.

Shallow stem wounds (from about 2 to 12 cells deep) were less efficient as infection courts than deep wounds (from one-fourth to completely through the stem) when the inoculum was identical. This result was attributed to the role played by chance in the distribution of the bacteria and to the larger amount of wound sap which favored the larger wounds as infection courts.

Ordinarily the ultimate size of the gall was correlated with the depth of the wound but was independent of the size of the initial inoculum. The largest galls observed resulted from inoculating deep stem wounds regardless of whether the inoculum was a single bacterium or large numbers of bacteria.

The relation (1) between tiny wounds, tiny galls, and few bacteria, (2) between shallow wounds, small galls and more bacteria, and (3) between deep wounds, large galls, and many bacteria was verified by isolation experiments.

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